

MAILLARD REACTION PRODUCTS IN PROTEIN AND
CARBOHYDRATE DIGESTION AND UPTAKE

Studies of interactions in vitro and in vivo

Rickard Öste



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av

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Title and subtitle MAILLARD REACTION PRODUCTS IN PROTEIN AND CARBOHYDRATE DIGESTION AND UPTAKE. Studies of interactions in vitro and in vivo.			
Abstract In this study the effects of Maillard reaction products on protein and carbohydrate digestion and absorption were examined in vivo and in vitro. A double-isotope technique was used to evaluate the effect of compounds formed in the Maillard reaction on the intestinal uptake of dietary proteins. It was found that low-molecular weight products from a glucose-lysine reaction mixture reduced the plasma level of dietary protein-derived lysine obtained in rats (100-120 g) after feeding. The effect was evidently the result of an interference with the intestinal protein digestion. The reaction mixture inhibited in vitro carboxypeptidase A (E.C. 3.4.17.1) and the brush border enzyme aminopeptidase N (E.C. 3.4.11.2). A glucose-lysine reaction compound, 2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine, was found to be a strong competitive inhibitor of aminopeptidase N ($K_i=0.2$ mM). When given to rats in an experimental diet (3 mg in 1 g diet), it reduced the level of lysine derived from both dietary free and protein-bound lysine and obtained in plasma three hours from feeding. This compound also inhibited carboxypeptidase A, as did a number of substituted furans and pyrroles. The inhibition did not follow classical enzyme kinetics, but could be described as an induced substrate inhibition. Nitrogen balance experiments on rats with fractions of a browned meat loaf crust indicated that water-soluble low-molecular weight compounds were not responsible for the low digestibility of this product. Low-molecular weight Maillard reaction compounds were found to be weak competitive inhibitors of lactase (E.C. 3.2.1.23), sucrase (E.C. 3.2.1.48), maltase (E.C. 3.2.1.10) and trehalase (E.C. 3.2.1.28). High-molecular weight Maillard reaction compounds ($M_w > 6,000-8,000$ daltons) were strong mixed type inhibitors of especially lactase and invertase. However, when intubated into rats they did not affect the hydrolysis or uptake of lactose or sucrose.			
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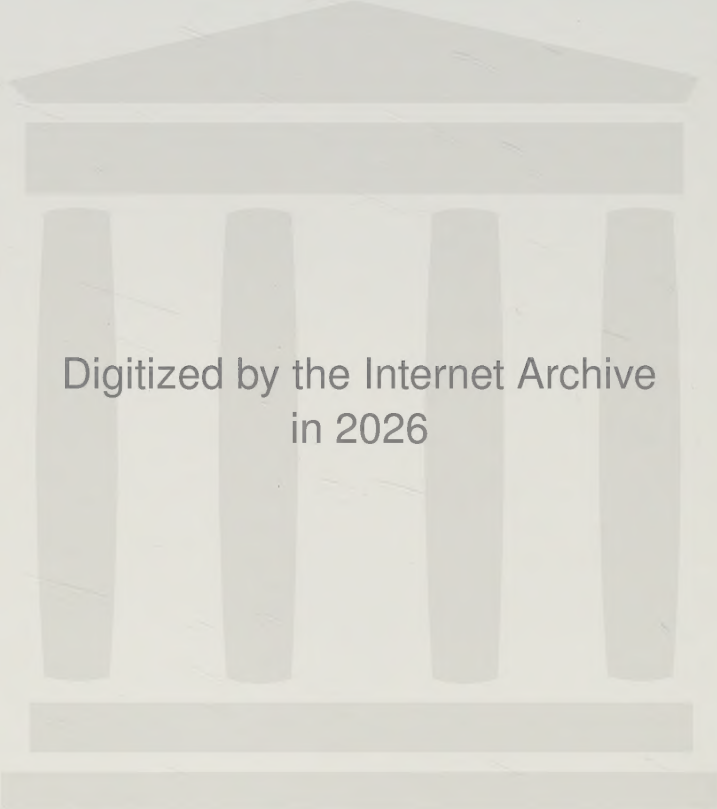
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This document is a summary of the following papers:

1. Goto, K. and Wada, T.
Effect of chemical reaction products on crystal growth in some systems
Journal of Materials. Submitted for publication.
2. Goto, K., Ishiguro, K., Wada, T., Saito, H. and Furuta, H.
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This dissertation is a summary of the following papers:

- I Öste, R. and Sjödin, P.
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In the text these papers will be referred to by their Roman numerals.

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INTRODUCTION

Protein and carbohydrate digestion and absorption

Proteolytic digestion and the subsequent uptake of breakdown products in the small intestine has been treated in a number of recent reviews (1 - 4). The present view of this process, with emphasis on the enzymatic hydrolysis and membrane transport, will be summarized below.

With regard to the origin of the gastro-intestinal proteolytic enzymes, and the site and nature of their action, the course of protein digestion may roughly be divided into five functionally different steps, i.e. a gastric phase, intestinal luminal hydrolysis, intestinal membrane hydrolysis, intestinal membrane transport and intracellular hydrolysis. Under optimal conditions the complete process will transform the dietary protein into free amino acids, which are the basic units in body protein synthesis. The extent to which these amino acids will in fact be used for protein synthesis depends, however, not only on the degree of digestion and absorption, but also on the status of the consumer and the composition of the absorbed amino acid mixture.

The gastric phase

In the first step, the proteins are attacked in the stomach by pepsin and hydrochloric acid. Pepsin is secreted into the stomach as a proenzyme and is activated in the acid milieu. It has a preference for peptide bonds involving phenylalanine, tyrosine or leucine. Due to its high specificity, the total action of pepsin on the dietary proteins is probably limited to the hydrolysis of a minor part of the peptide bonds, giving rise to polypeptides with preferentially N-terminal phenylalanine and leucine. The lowering of pH in the stomach, which is necessary for the activity of pepsin, will lead to a denaturation of the dietary proteins. Protein denaturation is known to facilitate the attack by proteolytic enzymes in vitro (5), probably due to the resulting random exposure of different peptide bonds (i.e. with various adjacent amino acids), thus offering peptide links even for rather specific endopeptidases. Thus the effect of HCl in the stomach will facilitate the digestion of previously undenatured dietary proteins in later stages of the digestion.

The contribution by the stomach to the total protein digestion is in general considered limited, at least as long as the subsequent digestion by the

pancreatic enzymes is not restricted (4, 6, 7). However, total gastrectomy may lead to a reduced protein uptake (7, 8). The mechanism behind this phenomena may be linked to an indirect effect of the stomach such as the fractional delivery of substrate to the small intestine and the effect of acid on pancreatic enzyme secretion (8, 9). If only the peptic digestion is reduced, the faecal nitrogen excretion remains unchanged (10). It should be observed in this context that most protein consumed in modern society is certainly denaturated before intake.

Intestinal luminal hydrolysis

In the second step of protein digestion, the denaturated proteins and larger peptides leaving the stomach and entering the small intestine, are attacked by the pancreatic enzymes. This will result in peptides of various length as well as a portion of free amino acids. The pancreatic proteases are trypsin, chymotrypsin, elastase (I and II) and carboxypeptidase A and B. They enter the duodenum from the pancreatic duct as inactive proenzymes and are activated by trypsin, which has first been activated by enterokinase in the intestine. The action of each of these enzymes is relatively limited, but their substrate specificities are complementary to each other and they should thus be synergistic in their synchronous action on dietary proteins. Observations made by Gertler et al (11) support this view. They inhibited individual enzymes, including the exopeptidases carboxypeptidase A and B, and found that, for any one of the enzymes inhibited, the combined action in vitro of the remaining pancreatic enzymes was reduced. The pancreatic enzymes are important in protein digestion, and the exclusion of pancreatic excretion in experimental animals or in man considerably enhances faecal nitrogen excretion (12, 13).

The digestion by the pancreatic enzymes leads to amino acids and smaller peptides which are assumed to have a size of 2 - 6 amino acids (14 - 17). It is unlikely that the pancreatic enzymes alone could achieve total digestion of the protein, and accordingly a significant amount of the dietary protein will still be in the form of peptides that require further digestion.

Intestinal brush border hydrolysis

In the intestine there are a number of membrane-bound as well as cytoplasmic peptidases in the epithelial cells (18, 19). These enzymes may be liberated into the lumen of the intestine as a result of cell desquamation, or they may

be active in the intact intestinal brush border. The contribution by the free luminal peptidases to the digestion of exogenous proteins is probably small (1). The action of the peptidases of the brush border may be considered as the third step of protein digestion. Since only free amino acids, dipeptides, and, possibly, some tripeptides are actively transported across the intestinal brush border (see below), all higher peptides have to be further hydrolysed prior to uptake. This third step of protein digestion can be described as the action of enzymes of the brush border that are not strictly dipeptidases. Enzymes of this type that have been isolated are aminopeptidase N, aminopeptidase A and dipeptidyl peptidase IV. The most abundant of these is aminopeptidase N, and in vitro experiments suggest that this enzyme contributes significantly to the total capacity of the brush border to hydrolyse peptides (20 - 23). The capacity of these brush border enzymes to hydrolyse longer peptides during in vivo conditions is evident from experiments performed with excluded pancreatic digestion. Although this significantly reduces the protein digestion, a considerable portion of the protein is still absorbed (see ref. 7 and references therein).

Intestinal membrane transport

The active uptake of free amino acids through the brush border into the epithelial cells is performed by at least two independent transport mechanisms in man. One of them transports monoamino-monocarboxylic neutral amino acids and the other transports dibasic amino acids and cystine. Studies in animals have given evidence for the existence of a third system, which transports dicarboxylic amino-acids. In addition to this, at least one transport system for unhydrolysed dipeptides has been found. Whether one or more systems exist is still a matter of debate, but the quantitative importance of the uptake of intact dipeptides is well recognized (1, 3, 4). The importance of absorption processes other than free amino acid transport has been proven by studies of partial protein hydrolysates and amino acid mixtures in rat and in man (24, 25). Results indicate that even intact tripeptides may be at least partly absorbed (2). Higher oligopeptides on the other hand, are not absorbed but have to be further hydrolysed before uptake.

Intracellular hydrolysis

The final step in protein digestion takes place inside the epithelial cells, where absorbed dipeptides and possibly tripeptides are hydrolysed to amino

acids (18). These intracellular enzymes apparently have sufficient capacity to handle incoming peptides in amounts that exceed the absorption of free amino acids (1, 3).

Carbohydrate digestion

The events occurring during the digestion and absorption of dietary carbohydrates are more easily understood than the process of protein digestion. For a recent review see ref. 26. The major food carbohydrate in most human diets is starch, which is composed of amylose and amylopectin. The hydrolysis of starch is catalysed by α -amylase excreted by the salivary glands and pancreas. This enzyme cleaves the 1, 4-glucoside linkage but leaves the 1, 6-glucoside bond of amylopectin intact. The products are maltose, maltotriose and limit dextrins. These are subsequently hydrolysed into glucose by amyloglucosidase, isomaltase and maltase present in the brush border of the intestinal epithelial cells. Sucrose is hydrolysed by brush border sucrase and lactose by brush border lactase. The mushroom disaccharide trehalose is split by trehalase. The monosaccharides formed in these processes, glucose, fructose and galactose, are actively transported across the brush border, which has one pathway for the transport of glucose and galactose, and another one for fructose.

Heating of foodstuffs and the Maillard reaction

Which effect the heating of the food will have on its different constituents depends essentially on the degree of the time-temperature treatment. Boiling of food at atmospheric pressure will denaturate protein, gelatinize starch, broke cellwalls and melt fat. These alterations, although quite obvious to the senses, do not in general imply by any chemical reaction forming new covalent linkages, but are essentially rearrangements of the threedimensional structure of the polymeric constituents of the food. From a nutritional point of view these changes have probably no adverse effect but will instead facilitate the digestion of starch and sometimes of the protein. When the temperature exceeds 100°C, which is concomittant to the loss of water, the well-known rapid browning of the surface starts. This browning is essentially caused by the non-enzymatic browning reaction between proteins and carbohydrates.

This complex reaction, named after the French chemist Louis-Camille Maillard, leads to the formation of numerous low-molecular weight substances as well as complex polymers. With the exception of the initial steps, the details of the reaction pathways are only fragmentarily known and are still the subject of basic research efforts. The chemical nature as well as various implications of the reaction have been the subject of a number of recent review articles and books (27-30).

The first step is generally agreed to involve the reaction (condensation) of an amino-compound (in foodstuffs this could be the α -amino group of free amino acids or the side chain of the protein-bound basic amino acids, especially the ϵ -amino group of lysine) with the carbonyl-carbon of a reducing sugar, to form a Schiff's base. This Schiff's base is rearranged to the more stable ketosamine, in the case of aldoses through the Amadori rearrangement, or to an aldosamine, when the reacting sugar is a ketose. These reactions have been referred to as 'early Maillard reactions' (31) and are believed to be the initial key steps through which the precursors to the ultimate reaction products are formed. These amino-sugars may easily dehydrate to unsaturated, reactive compounds, which through a number of reaction mechanisms, including reversed retro aldol-condensation, fission of dicarbonyls, fragmentation, Strecker degradation and combination, may form a number of aliphatic as well as aromatic and heterocyclic compounds. These steps have been referred to as 'advanced Maillard reactions' (31) and the products formed as 'premelanoidines' (32).

As the reaction proceeds further, strongly coloured, high-molecular weight compounds are formed and may precipitate. They are often named 'melanoidines' (32) as the result of 'final Maillard reactions' (31). The primary structure of these polymeric substances is largely unknown.

The reaction rate is dependent on temperature and on water activity. In comparatively dry food systems (a_w 0.4-0.7) the reaction may take place at temperatures well below 100°C (33, 34) the extent being in practise largely dependent on the amount and type of reducing carbohydrate present (35, 36).

Some of the formed compounds give the characteristic flavour of fried or baked food and are volatile, some are consumed with the food and contribute to the fried or burned taste (37, 38).

The biological fate of Maillard reaction compounds have been studied in rats. The low-molecular weight products of model reaction mixtures of glucose and lysine and of glucose and casein are absorbed in the small intestine and, to a large extent, excreted in the urine (39, 40). The high-molecular weight compounds are not absorbed, but are recovered, partly degraded, in the faeces (41).

There are a number of possible physiological implications of the Maillard reaction in food. Firstly, the reaction may consume the essential amino acid lysine and may lower the protein quality. This effect has been extensively studied (see ref. 42 and 43) and is probably the most important of the mechanisms impairing the quality of food proteins during processing. Secondly, the formation of anti-nutritional or toxic substances have been suggested (32). Clearly, the multiplicity of compounds that may be formed makes it difficult to exclude the possibility of the formation of some with adverse biological effects. A few long-term studies on rats with diets containing mixtures of Maillard reaction products have been performed, indicating these to be generally non-toxic (44, 45). However, the type and the amount of various different compounds present in these diets are unknown and this limits the value of generalizations based on these observations. Thirdly, the formation of mutagenic compounds during conditions favouring the Maillard reaction have been reported (46). The practical significance of these observations remains to be clarified.

In consequence with the reactivity of the ϵ -amino group of lysine in the Maillard reaction, the heating of food proteins together with carbohydrates may thus lead to a reduced nutritional value of the protein. This has recently been reviewed by Mauron (31) and by Dworschak (47).

The degree of this reduction is dependent on a number of factors, including water activity, type and amount of reducing sugar, type of protein as well as temperature and time of the heat treatment. The loss of protein quality may be expressed as reduced biological value (BV) and/or digestibility (TD) as apparent in a conventional net protein utilization (NPU) assay with rats. When the heat treatment is mild, a loss in the BV often corresponds to the loss of biologically available lysine caused by the Maillard reaction, provided lysine is the limiting amino acid in the protein. When the heating is more intense, the reduction of BV may be greater than the apparent loss of available lysine and there may also be a reduction in TD.

There are some reports on possible mechanisms behind these effects of severe heat treatment. Adrian (32) has shown that water-soluble premelanoidines from a glucose-glycine reaction mixture reduce the protein digestibility and also affect the utilization of absorbed amino acids. Valle-Riestra (48) suggests that the reduced uptake of a severely heated glucose-egg-albumen mixture is the effect of a decreased pancreatic enzyme secretion. The observed decrease in BV (i.e. enhanced urinary nitrogen excretion) may also be explained by the uptake of non-metabolizable nitrogen-containing compounds from the gut (48, 49, 50).

Thus, in addition to the losses of available lysine, there may be other effects of the Maillard reaction on the protein utilization. The mechanisms behind such interactions are, however, less well known.

Objectives of the present investigation

The aim of the present study was to examine how compounds formed during the Maillard reaction per se might influence the digestion and uptake of proteins and carbohydrates. The overall picture of protein digestion thus indicates a flexible mode of action, with no indispensable key step, but with a number of enzymic functions that may be interferred with. Because of the complexity of the proteolytic process, the design of the study was to first study effect in vivo, and when an effect was observed, to continue to study effects in vitro on various steps of the digestive process.

The number of different Maillard reaction compounds that may be found in a specific foodstuff is probably overwhelming (27-30). Therefore, the experiments were initially performed with a model reaction mixture of glucose and lysine rather than with individual compounds.

The chemistry of the reaction system glucose-lysine was simultaneously studied at the Department of Chemistry and Molecular Biology, SLU, Uppsala, Sweden. This circumstance made possible the subsequent effort to link some of the observed effects to specific compounds of the Maillard reaction.

METHODS

Model Maillard reaction mixture

In in vivo and in vitro experiments (I, II, IV), a glucose-lysine reaction mixture was used as a model of Maillard reaction products. This was obtained by dissolving equimolar amounts of glucose and lysine in distilled water and boiling the solution under reflux for 24 hours. The pH was kept constant during the reaction through pH-stat controlled addition of 5N NaOH. The pH-stat was set at pH 6.5. After the reaction the pH of the mixture at room temperature was, however, about 5.3. The reaction mixture was dialysed against distilled water in a dialysis bag with an exclusion limit of 6000-8000 dalton. The dialysate was concentrated and unreacted glucose was removed through column chromatography on ConA-Sepharose. The resulting glucose free eluate was concentrated and constituted the low-molecular weight (LMW) fraction used in the study. The retentate from the dialysis was centrifugated to remove insoluble material and then concentrated by evaporation. This material constituted the high-molecular weight (HMW) fraction in the study.

Animal experiments

Male Sprague-Dawley rats were used in all experiments. They were kept in (25°C) and humidity (RH 50%) controlled rooms before and during experiments and were given stock laboratory diet as food when not subjected to experiments. The rats were obtained from Anti Cimex, Sweden, and were allowed to adapt to their new environment for at least two days before the experiment.

The NPU experiments were performed as described earlier (51) with the following modification: Ten per cent sucrose was added at the expense of starch to improve the palatability of the diet. Nitrogen was determined by the Kjeldahl method.

When assessing the nature of a protein quality reduction, the NPU-method has certain limitations. For example, a reduced TD is not necessarily the result of reduced digestion of dietary protein, since obviously the same result will be obtained if the increase in faecal nitrogen is caused by an enhanced excretion of endogenous nitrogen or if there is a fixation of metabolic nitrogen by the colonic flora. To be able to study specifically the digestion of an exogenous protein in rats, a methodology was developed as follows: A solution of a radioactive amino acid ($U-^{14}C$ -lysine, $3,4-^3H$ -lysine or ^{35}S -cystine) was injected into the wing vein of a laying hen at the time of

rapid egg-albumin synthesis. The eggs were recovered, the egg-white separated and dialysed to remove glucose and then lyophilized. Based on autoradiographic studies on TLC separated acid hydrolysates of the egg-white protein, it was concluded that the radioactivity present was derived almost exclusively from protein-bound lysine when labelled lysine was injected. Of metabolic reasons (the metabolism of cystine, see ref. 52) the ^{35}S label injected in the form of cystine was to be found exclusively in the cystein moieties, when protein-bound.

These labelled proteins were then used to prepare diets that contained free and protein-bound differently labelled lysine or, on one occasion, protein-bound ^{35}S -cystine and protein-bound 3,4- ^3H -lysine. A portion of the diet was given to fasting rats (weight 100-120 g), early in the morning, to be consumed ad libitum. We have observed that most rats after one night's fast will in the morning immediately consume at least 2 g of food, provided the animal room is kept dark and the food is not too untasty. In these experiments the rats, with few exceptions, completely consumed the portion within 15 min. When this did not occur, the rat was omitted from the study. After a well defined time after finishing the meals, each rat were anaesthetized with diethyl ether, blood was collected by heart puncture, and the radioactivity in plasma was measured.

The effects of the addition of Maillard reaction products to the diets on the levels and on the ratio of different radioactivities were then measured to detect specific *in vivo* digestion effects.

To study the effect of Maillard reaction products on the *in vivo* uptake of carbohydrates the methodology as described by Dahlqvist et al was followed (53, 54). This procedure includes intubation of a solution of the carbohydrate to be studied into the stomach of 150-180 g fasting male rats, followed after various times by the quantitative analysis of remaining carbohydrates in the recovered gastro-intestinal tracts.

Enzyme experiments

The effect of the LMW fraction on the activities of pepsin, trypsin, chymotrypsin, carboxypeptidase A and carboxypeptidase B was studied with commercially obtained enzymes. The effect on brush border peptidases was studied with preparations of microvillus membranes from pig intestine as described by Sjöström et al (21), using enzyme specific substrates. Kinetic experiments on aminopeptidase N were performed using the pure enzyme, prepared as described by Sjöström et al (21).

The effect of the LMW and the HMW fraction on salivary amylase was studied with α -amylase from human saliva, and the effect on maltase, sucrase, lactase and trehalase was studied with perorally obtained biopsy specimens of human small intestine mucosa (55).

Miscellaneous

Liquid-scintillation counting of ^{14}C , ^{35}S and ^3H (I, II, III) was performed in a Searle Mark III apparatus with scintillation fluids from the Packard Instrument Co. External standard-channel ratios were used to determine the counting efficiency.

Analysis of carbohydrates (IV) were performed either by measuring total reducing power, after enzymatic cleavage, with DNS reagent (56) or directly with the anthron reagent, which measures both free and bound hexoses.

Synthetic compounds not purchased (III) were prepared by Raya Miller at the Department of Chemistry and Molecular Biology, SLU, Uppsala, Sweden. Methods used were essentially as described in the literature.

RESULTS AND DISCUSSION - PROTEIN

Studies in vivo with the model Maillard reaction mixture

The addition of 1.5% of the low-molecular weight (LMW) fraction of the Maillard reaction mixture to the rat diet containing free ^3H labelled lysine and protein-bound ^{14}C -labelled lysine resulted in a statistically significant reduction (13%) in the ratio of ^{14}C lysine to ^3H lysine in plasma measured two hours after feeding (I). Before the interpretation of these results a brief account of the mechanisms that regulate the level of free amino acids will be given together with a comment on the methodology used.

After the intake of a protein containing meal, the level of amino acids in the portal vein rises. A rise in the level of amino acids in systemic blood will also be observed, but this will be smaller. The influx of certain free amino acids will stimulate protein synthesis and result in a reduction in the free amino acids in the plasma (57). The absolute level of a certain amino acid in the rat plasma after a meal will, at any moment, depend on a least, 1) the amount of dietary protein, 2) the amino acid composition of the dietary protein, 3) the digestibility of the dietary protein, 4) the amount and composition of other food constituents, 5) the rate of stomach emptying, 6) the rate of amino acid absorption, 7) metabolism of amino acids in the intestine, 8) the time elapsed after the intake of the meal and 9) the nutritional, physiological and psychological status of the rat (58, 59). In the present experiments equivalent groups of rats were given diets with equal composition except for the addition of the Maillard reaction compounds under study. Thus any effect of this addition upon the level of amino acids in plasma should be monitored through an alteration in 3), 5), 6), 7), or 9).

The level of amino acid was recorded as radioactivity in plasma. This radioactivity may in principal come from amino acids, from metabolites or from newly synthesised proteins. However, Dawson and Porter (60) performed studies on rats and cats fed with ^{14}C -labelled proteins and found that the radioactivity of portal and systemic plasma was almost exclusively due to free amino acids even after 3 hours. They also observed that the peak activity was to be found 1-2 hours after ingestion.

The amino acids in plasma are partly recycled to the intestine in the form of digestive enzymes. However, it is unlikely that any appreciable amount of the amino acid activity in the two hour plasma originates from labelled endogenic protein, since, on the one hand, there is a certain turnover time

for these proteins (about 50 min in the pancreas, (61)) which delays the labelling, and, on the other hand, the digestion of these proteins most likely occurs after the digestion of exogenous protein (7, 62). Thus it is reasonable to assume that the radioactivity observed in plasma two to three hours from feeding reflects the level of amino acid in plasma derived from the diet.

As mentioned above the effect observed upon the addition of the LMW fraction was a statistically significant reduction in the ratio of label originating from protein-bound lysine and from free lysine in the diet. The mean of the absolute level of the ^3H label (from free lysine in the diet) was the same in both groups (I). The mean of the ^{14}C label tended to be lower in the group fed with the LMW fraction, but the difference was not statistically significant. From the coefficients of variation in these measurements it can be concluded that a greater number of rats has to be studied in order to show significance in the differences in the absolute levels. The study of the ratio of levels, which showed a lower coefficient of variation, evidently reduces the influence of "biological variations" on some of the factors affecting the plasma levels of amino acids. The observed effect on the ratio of the labels focused the attention on the protein digestion and on stomach emptying, since any effect on amino acid transport, intestinal amino acid metabolism and nutritional/physiological status of the rat should affect both labels.

The effect of the addition of the LMW fraction on the progress of both labels in plasma was studied in a separate experiment where blood samples were taken from rats sacrificed after various time intervals (I). The results showed a similar initial rise in label levels in both groups and a tendency towards a lower dietary-protein-derived ^{14}C level but not the dietary free-amino acid-derived ^3H level after two hours and onwards. This indicated that the course of stomach emptying was the same in both groups and that the effect of the LMW fraction was mediated through an interference with the protein digestion or, possibly, with the peptide absorption.

In another experiment diets containing protein labelled with ^{35}S -cystein and ^3H -lysine were given with and without the addition of the LMW fraction (I). The ratio of the labels in two-hour plasma was the same in both groups. This observation indicates that effects of the LMW fraction on the digestion and/or peptide uptake of dietary proteins were similar for different protein-bound amino acids.

The addition of the HMW fraction to the diets resulted in a small but significant increase in the $^{14}\text{C}/^3\text{H}$ ratio, i.e. there were comparatively more of

the originally protein-bound lysine in the plasma. This effect was not further investigated.

Studies in vitro with the model Maillard reaction mixture

The effect of the LMW fraction on the in vivo uptake of proteins may be directly on the enzyme action in the intestine or indirectly on the formation of proteolytic enzymes. To study whether a direct effect could be assumed, the activities of a number of gastro-intestinal enzymes were assayed in the presence of low concentrations of the LMW fraction (II). Of the enzymes tested, a significant inhibition was observed only with carboxypeptidase A and aminopeptidase N. The inhibition of carboxypeptidase A could not be fitted to the classical equations of enzyme kinetics and inhibition (63). The inhibition of aminopeptidase N was of the mixed type.

The significance of these findings in relation to the observations in vivo cannot be unequivocally stated since, naturally, the conditions in vitro differ from those in vivo. However, the inhibition of aminopeptidase N might be crucial, since this enzyme may have a key role in the final digestion of proteins (see INTRODUCTION).

The LMW fraction was further fractionated through column chromatography and the inhibitory capacity of the subfractions on the activities of carboxypeptidase A and aminopeptidase N was assayed (II). This revealed that a number of inhibitors of both enzymes were present in the Maillard reaction mixture. One fraction that was strongly inhibitory to both enzymes was found to contain 2-formyl-5-hydroxymethylpyrrole-1-norleucine, a compound isolated from a glucose-lysine reaction mixture by Miller et al (64).

Studies in vitro with single Maillard reaction compounds

Synthetic 2-formyl-5-hydroxymethylpyrrole-1-norleucine was found to strongly inhibit carboxypeptidase A and aminopeptidase N. The effects of a number of chemically related compounds were assayed in order to uncover the typical structures of Maillard reaction products that were inhibitors (III). Various substituted furans and pyrroles inhibited carboxypeptidase A. The strongest ones were furans with a carboxylic group as substituent. 5-(hydroxymethyl)-2-furoic acid and 2-furoic acid, which were strong inhibitors, are probably formed by oxidation of 5-(hydroxymethyl)-2-furaldehyde (HMF) and 2-furaldehyde, respectively (65). HMF is one of the major Maillard reaction compounds that is formed from hexoses, especially under weakly acid con-

ditions. 2-furaldehyde is formed from pentoses by the equivalent mechanism.

Some N-unsubstituted pyrroles as well as the N-substituted 2-formyl-5-hydroxymethylpyrrole-1-norleucine were also inhibitory to carboxypeptidase A. The latter compound was the strongest inhibitor of aminopeptidase N (Ki 0.2 mM). Only a few additional, weak inhibitors of aminopeptidase N were found. They were all competitive to the assay substrate L-alanyl-p-nitro-anilide and had a carboxylic group as substituent. Since the LMW fraction was a mixed type inhibitor of aminopeptidase N, a systematic search for further inhibitors was performed on fractions from a glucose-lysine reaction mixture that had previously been investigated from a chemical point of view by Miller et al (66). In spite of the considerable effort put into this investigation, no mixed-type or non-competitive type of inhibitor was found.

N-unsubstituted pyrroles are assumed to be formed via a reaction pathway including Strecker degradation of free amino acids (67). Since the major reactive amino groups of most foodstuffs are those of the protein (mainly the δ -amino group of lysine and the terminal α -amino group) this should instead favour the formation of N-substituted compounds that may be linked to the protein. Studies by Olsson and coworkers (69, 70) on the reactions of glucose with methylamine and glycine indicate that, when the Strecker degradation cannot take place, the formation of N-substituted compounds is favoured. Thus 2-formyl-5-hydroxymethylpyrrole-1-norleucine may be formed from protein-bound lysine. Experiments are in progress to evaluate this possibility. If this compound exerts an effect on the enzymes while bound to proteins or to peptides remains to be studied.

Studies in vivo with single Maillard reaction compounds

The effect of two potent inhibitors on the in vivo uptake of proteins was evaluated with the double-isotope technique described above. The addition of a minute amount of 2-formyl-5-hydroxymethylpyrrole-1-norleucine to the diet (3 mg/g) resulted in a small (10%) but statistically significant reduction in the absolute level of activity of both isotopes (originating from free and from protein-bound lysine in the diet) in the three-hour plasma. However, no significant difference in the ratio between the labels was observed. This result might be explained by a reduction in the intestinal protein digestion and/or peptide uptake in addition to a reduction in the absorption of free lysine, bearing in mind that a significant portion of the digested protein is probably absorbed via the peptide transportation system(s), (see INTRODUCTION).

Another possibility that cannot be excluded is an effect of the added compound on the metabolism of absorbed lysine.

The addition of 2-furoic acid (3 mg/g diet), which was a strong inhibitor of carboxypeptidase A but a very weak inhibitor of aminopeptidase N, had no statistically significant effect on the plasma levels of radioactivity.

Nitrogen balance studies on fractions of a meat loaf crust

NPU studies were performed to evaluate whether water soluble low-molecular weight substances present in the crust of a fried meat loaf may interfere with the utilization of protein by the rat.

The crust was extracted with water, and the residue, the water extract added to the crumb, the intact crust and the intact crumb were assayed. The results show that, compared with the crumb, the reduced digestibility of the crust could not be explained by the presence of deleterious compounds in the water extract.

The content of reducing carbohydrates in the meat loaf should be rather low, and, accordingly, the presence of enzyme inhibiting compounds of the types described above may have been very low. Thus the results do not exclude the effect of such compounds in other types of food, but they indicate that in meat loaf crust other mechanisms are of importance for the low digestibility. These may include the formation of cross-links in the peptide chains (70).

RESULTS AND DISCUSSION - CARBOHYDRATES

Studies in vitro

The results observed with the proteolytic enzymes motivated an examination of the enzymes involved in the carbohydrate digestion. Assays of α -amylase and the intestinal disaccharidases together with various amounts of both the low-molecular weight (LMW) and the high-molecular weight (HMW) fraction from the glucose-lysine reaction mixture showed that both of them were inhibitory to the disaccharidases but not to the amylase (IV). The LMW fraction was a weak competitive inhibitor and the HMW fraction a strong mixed-type inhibitor of especially lactase and sucrase.

Various polyols are known to be competitive inhibitors of disaccharidases (71, 72). Especially the early Maillard reaction products to be found in the LMW fraction have polyolic structures and this may explain their inhibitory capacity. However, since the inhibition was weak and the practical significance of this effect is probably limited.

The strong effect of the HMW fraction was both of competitive and non-competitive nature. The complete structures of these polymeric Maillard reaction compounds are unknown. However, the presence of both hydroxy groups and more dehydrated, aromatic parts have been detected (65, 73). The compounds are also known to be strongly absorbed by protein (74). Thus, the HMW fraction have features that could account for both types of inhibitions observed.

Studies in vivo

The effect of the HMW fraction on the uptake of sucrose and of lactose was studied in rats (IV). The addition of the HMW fraction in a ratio of 1:100 to the carbohydrates had no effect on the absorption. Increasing the ratio to 1:10 in the lactose solutions still had no effect on the uptake. Earlier observations (40) and a report in the literature (75) indicate that polymeric Maillard reaction compounds may remain in the stomach a considerable time after ingestion. They may, in the present study, thus have been separated in the stomach and the lactose would have been hydrolysed and absorbed unaffected. To circumvent this possibility, the HMW fraction was administered at various times in advance of the lactose solution. However, the lactose absorption still proceeded unaffected.

These results were somewhat surprising since it is known that the lactase activity, i.e. the hydrolysis of lactose to glucose and galactose, is the rate limiting step in the uptake of lactose from the intestine both in rat and in man (54, 76). The reason for the discrepancy between the in vitro and the in vivo experiments remains to be investigated.

CONCLUSIONS

The conclusions based on the results of the present study may be summarized as follows:

1. Compounds formed in the Maillard reaction may affect the utilization of dietary protein in rats. This effect may be the result of an interference with the enzymes involved in the digestion and uptake of proteins in the intestine, especially aminopeptidase N of the brush border.
2. Some furans and pyrroles formed in the Maillard reaction are strong inhibitors of carboxypeptidase A. The inhibition is especially strong when a carboxylic group is a substituent on the furans.
3. An N-substituted pyrrole, 2-formyl-5-hydroxymethyl-1-norleucine, is a competitive inhibitor of aminopeptidase N. This compound is formed in the glucose-lysine reaction.
4. Water-soluble compounds extracted from a meat loaf crust do not interfere with the protein digestion in rats, when tested in a concentration equivalent to those in the intact crust.
5. Low-molecular weight Maillard reaction compounds are weak, competitive inhibitors of lactase, sucrase, maltase and trehalase.
6. High-molecular weight Maillard reaction compounds are strong mixed-type inhibitors of at least lactase, invertase and maltase.
7. In spite of the inhibition of disaccharidases in vitro, the high-molecular weight Maillard reaction compounds do not interfere with the uptake of lactose or sucrose in the rat.

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EFFECT OF MAILLARD REACTION PRODUCTS ON PROTEIN
DIGESTION. IN VIVO STUDIES ON RATS.

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Short title: Maillard reaction products and protein uptake

ABSTRACT

The effect of glucose-lysine reaction mixture on the protein digestion in rats was studied, using ^3H -labelled free lysine, ^{14}C - or ^3H -labelled protein-bound lysine and ^{35}S -labelled protein-bound cystine in the experimental diets. The low-molecular-weight part of the glucose-lysine reaction mixture (1.5% w/w in the diet) affected the utilization of dietary protein-derived amino acids, as revealed by the ratio and the levels of different labels in plasma after feeding.

The browned crust from a heated minced-meat loaf was less well digested and had a lower biological value than the crumb in a nitrogen-balance study with rats. When the water-soluble fraction of the crust was removed, the biological value of the crust was restored to the value of the crumb but the digestibility remained low. The addition of the water-soluble fraction of the crust had only a slight effect on the digestibility of the crumb.

It is concluded that compounds in the glucose-lysine reaction model affects the dietary protein utilization of rats but were not present or otherwise could not explain the reduced utilization of proteins in the crust of the heated meat product.

Key words: Maillard reaction - protein digestion - nitrogen balance.

It is generally recognized that processing and storing of foodstuffs may cause a reduction in the quality of the constituent proteins. The nature and degree of this effect depends on the original composition of the food item and the type of treatment. When reducing carbohydrates are present, conditions are in favour of the Maillard reaction (for a review of various aspects on the Maillard reaction in food, see 1). When such foodstuffs are mildly heated, a decrease in protein quality is primarily attributed to the reaction of the epsilon-amino group in lysine with the reducing part of the carbohydrates present, leading to the formation of a physiologically unavailable product. With more pronounced heat treatment, however, the loss of protein quality cannot be explained solely by the observed reduction of the amount of lysine (2). An effect on both biological value (BV) and digestibility may be observed in a biological assay (3, 4). A reduced BV may at least partially be explained by the intestinal uptake and urinary excretion of unavailable peptides (4, 5).

A possible, indirect effect of the Maillard reaction on the protein utilization is suggested by Adrian (6), who reported that the water-soluble fraction of a glucose-glycine reaction mixture reduced the digestion of casein *in vitro* and *in vivo* on rats and also increased the urinary nitrogen excretion.

The present report is the first part of a study on the effect of compounds in a glucose-lysine reaction mixture on the protein digestion and absorption. A double isotope technique was used to study the digestion of dietary protein

in vivo on rats. The results indicate the possibility that low-molecular-weight reaction compounds have a depressing effect on the uptake of amino acid from dietary protein. An observed reduction in digestibility of a meat crust compared to the crumb could, however, not be explained by the presence of low-molecular-weight water-soluble compounds in the crust.

EXPERIMENTAL

Glucose-lysine reaction mixture

A glucose-lysine reaction mixture was prepared by reflux-boiling 4.50 g D-glucose with 4.55 g L-lysine HCl in 100 ml aq. dest. The pH was kept at 6.5 throughout the reaction by a pH-stat controlled addition of 5N NaOH. After the reaction, the pH measured at room temperature in repeated experiments was in general lower, about 5.5.

The reaction mixture was repeatedly dialyzed against aq. dest until an almost colourless dialysate was obtained. The dialysis tube (Spectrum Medical Industries, Los Angeles) had a molecular weight cutoff of 6.000-8.000 daltons. The combined dialysates were dried by evaporation, dissolved in a small amount of 0.1 M ammonium acetate buffer, 1 mM in Ca^{2+} , Mg^{2+} and Mn^{2+} , and applied on a column packed with ConA-Sepharose (Pharmacia, Sweden) to remove remaining free glucose. The eluate was concentrated by evaporation. This fraction contained the low-molecular-weight (LMW) reaction compounds. The yields were 7.4-7.7 g dry substance.

The retentate from the dialysis was centrifuged to remove insoluble material, and concentrated. This fraction contained

the soluble high-molecular-weight (HMW) compounds. Yields were 0.9-1.2 g.

Radiolabelled egg-white protein

1 mCi 1-(U- ^{14}C)-lysine, 1 mCi ^{35}S -cystine or 5 mCi 1-(4,5- ^3H)-lysine (from Radiochemical Centre, Amersham GB) were injected into the wing vein of 24-26 weeks old laying hen of the Shivar strain, 1-2 hours before laying. The eggs were collected and the egg whites were separated, dialyzed against aq. dest to remove glucose, and lyophilized.

Aliquots of ^{14}C -labelled egg white were hydrolyzed with 6N HCl for 20 hours at 110°C . A suitable amount of each hydrolyzate was applied to a thin layer plate and the lysine was separated from the other amino acids through elution with chloroform/methanol/17% NH_3 (2:2:1: v/v/v). The plates obtained were examined by autoradiography, revealing that only trace amounts of radioactivity were from non-lysine residues in the egg-protein. The ^{35}S of injected cystein could only be present in the egg protein as cystein/cystine label.

Meat crust and crumb

Minced-meat loaf made from rolled rump (32%), rindless ham (32%), rindless pork back fat (11%), bread crust powder (4.5%), potatoe powder (2.5%), egg-white powder (1%), salt and seasonings was baked for 30 min in a forced convection oven at 225°C with an air velocity of 5 m/s, giving a final temperature of 70°C in the centre. The separated crust (13% of the dry weight of the loaf) and the crumb were homogenized and lyophilized. Part of the crust preparation was extracted with water by ultrasonication for 10 minutes. Both the extract

(16.3% of the crust) and the insoluble residue were recovered and lyophilized. Amino acid analysis were performed at the Central Amino Acid Analysis Laboratory, BMC, Uppsala, Sweden.

Rat experiments with radioactive diets

For these experiments the casein diet used in the nitrogen balance assay (see table 1) was mixed with lysine- ^{14}C -labelled egg-white protein (98:2 w/w). The specific activity of the egg-white protein was 32,000-46,000 dpm/mg. In addition, a small amount of free $4,5$ - ^3H -lysine (80 Ci/mmol) was added, giving a ratio of ^{14}C dpm to ^3H dpm of approximately 0.1.

To test the influence of the LMW fraction, this was in some experiments added to the radioactive diet giving a concentration of 1.5% (w/w).

In one experiment 1.7% (w/w) of the HMW fraction was added.

Further one diet was prepared to contain double labels in the protein, namely both $4,5$ - ^3H -lysine-labelled egg white and ^{35}S -cystein-labelled egg white. The ^3H -labelled egg-white used for this diet gave 59,000 dpm/mg and the ^{35}S -labelled 12,000 dpm/mg. In order to keep the protein content constant, most of the casein had to be replaced by radioactive egg white to obtain good activity of ^{35}S together with an excess of ^3H .

For the animal experiments we used male Sprague-Dawley rats, weighing 100-120 g. They were fasted overnight. In the morning they were fed 1.5 g of the diet (1.0 g in the experiment with double labelled proteins) which they consumed within a few minutes. Two hours later they were anaesthetized with diethyl ether and blood samples, taken by cardiac punctation, were

put into heparinized tubes. The plasma was separated by centrifugation, and the radioactivities of ^3H and $^{14}\text{C}/^{35}\text{S}$ were measured by scintillation counting after mixing with Instagel scintillation fluid (Packard Instr. Co., Ill). In a separate experiment blood samples were taken at 45, 90, 135, 180 and 240 minutes after feeding.

Rat assay of protein quality

True digestibility (TD) and biological value (BV) were determined with growing rats (Sprague Dawley), five per assay, as described by Eggum (7) with the diet compositions as shown in table 1. Nitrogen was determined by the Kjeldahl method. A casein control, crust and crumb of the meat loaf were assayed. In addition, crumb was assayed when mixed with the water-soluble extract of the crust in the same proportion as in the crust. The insoluble residue of the extraction was assayed as well.

RESULTS

Table 2 shows the ratios of ^{14}C (from egg-white) to ^3H (from free lysine in the diet) found in the two hour plasma of the rats fed the radioactive diets with or without the LMW or HMW fraction. Compared to the control diet, a significant reduction of the ratio by 13% was observed in the group fed the diet containing the LMW fraction. In the group fed the diet containing the HMW fraction, a small, but statistically significant increase in the ratio was noted. The absolute

levels of activity found in the plasma, also given in table two, were not significantly different from the levels of the control group. The coefficients of variation of the absolute values were considerably higher than the values calculated on the ratios.

The progress of the levels of ^{14}C and ^3H in the plasma of the rats receiving the diet with or without the LMW fraction are shown in figure 1. There was a constant rise of the activity of both isotopes in both groups during the first 2-3 hours. A plateau was then obtained. No clear difference in the level of ^3H between the two groups was observed and since the initial progress of the ^3H levels were similar, we assumed that the course of stomach emptying was the same in both groups. The plateau level of ^{14}C activity in the group fed the LMW fraction was somewhat lower than in the control group, indicating an effect on the turnover of amino acids from dietary protein, effecting the plasma level of ^{14}C even four hours after ingestion.

In table 2 is also shown the ratio of ^{35}S to ^3H (both labels protein-bound in the diet). No significant difference was observed between the group fed the diet containing the LMW fraction and the control diet. This indicated that the effect of the LMW fraction observed was probably not limited to lysine, but included other protein-bound amino acids as well.

In table 3 are shown results of the nitrogen balance experiments of the meat loaf fractions. The crumb was almost

completely digested and had a BV of 71. Both the TD and the BV of the crust were lower compared to the crumb.

Removal of the water-soluble fraction from the crust (i.e. assay of the insoluble residue) did not affect the TD but increased the BV to the level of the crumb. Upon the addition of this fraction to the crumb a very small reduction in the TD was observed. The obtained lowered mean of the BV was not statistically significant.

The contents of essential amino acids in the crust and in the crumb are shown in table . The only notable differences were a reduction by 12% of the sulfur amino acids and by 15% of lysine in the crust. In table 4 is also shown the rat amino acid requirement pattern. If the remaining lysine was completely available, the sulfur amino acids were limiting in both the crumb and the crust.

DISCUSSION

We chose to compare the ratios of activities in plasma obtained from the rats two hours after feeding, which is when the plasma level of lysine is highest (unpublished observation). In addition, we studied the effects of the LMW fraction on the progress of absolute levels of radioactivities in plasma. The observed statistically significant reduction in the two hours ratio together with the small but distinctly depressed progress of the absolute activity indicated a reducing effect of the LMW fraction on the utilization of lysine from dietary proteins. When two differently labelled protein-bound amino acids (lysine and cystine) were given, no difference in the plasma

ratio of the radioactivities was observed upon the addition of the LMW fraction to the diet. The results indicated a general effect of the fraction on the utilization of amino acids from dietary proteins.

The most likely explanation to these observations is that the LMW fraction interfered with the digestion of dietary proteins. If proteolytic activities in the intestine were inhibited, these would probably result in a diminished uptake of originally protein-bound amino acids in general but would not influence the uptake of free amino acids (e.g. lysine). One cannot exclude, however, that the effect is the result of an altered metabolism of the absorbed amino acids, affecting the different labels to a different extent. This is a possibility since the metabolism of the $4,5\text{-}^3\text{H}$ label and the $\text{U-}^{14}\text{C}$ label of lysine is not the same.

The coefficients of variation were greater for the absolute values of activity than for the corresponding ratios of the different labels. This observation merits the choice of criteria for effects on protein digestion used in this study, i.e. the plasma ratio of labels from dietary free and protein-bound amino acids rather than the absolute levels. In fact, the absolute levels of activity in these experiments did not show any statistically significant differences.

In a study of Adrian (6) an increased fecal excretion of nitrogen in rats was induced by the presence of water-soluble glucose-glycine reaction products in the diet. These products also impaired the *in vitro* digestion of casein by pancreatic enzymes. With the additional results presented here it thus seems reasonable to assume that one or several low-molecular-weight compounds formed during conditions favouring the Maillard reaction may in fact interfere with

the digestion of dietary proteins in the rat intestine.

The slight increase of the ratio $^{14}\text{C}/^3\text{H}$ observed in the plasma of rats receiving the high-molecular-weight (HMW) fraction may be the result of an altering in the rate of stomach emptying in this group. It was obvious from the study of the LMW fraction (figure 1) that the ratio of the labels in plasma changed with the time from feeding. However, no study on the progress of the labels in plasma was made with the HMW-fraction and the effect observed remains to be explained.

The results of the experiments with the meat loaf do not suggest that the low digestibility of the crust in fact is the effect of any water-soluble compound(s). The water-insoluble residue of the crust had equally low TD as the whole crust and the addition of the water-soluble fraction only slightly affected the digestibility of the crumb. The substantially reduced digestion of the crust can, on the other hand, not be explained by a general loss of amino acids, since the amino acid composition of the crust and the crumb were almost equivalent and the BV of the water-insoluble residue was equal to the BV of the crumb. It is known that protein-crosslinks may be formed during severe heat treatment (9, 10). To what extent such links were formed during our heating procedure we do not know. The possibility exists that, if present, they would have reduced the digestion.

Although not specifically investigated, another factor of importance may simply be the change in physical state of the proteins upon heating, which make them less soluble (11)

and perhaps less susceptible to the digestive enzymes. It was obvious from the grinding and preparation preceeding the analysis that the crust exhibited physical properties different to those of the crumb.

When the water-soluble fraction was removed from the crust, its BV increased significantly. Adrian (6) has reported that the presence of water-soluble glucose-glycine reaction compounds in the diet increased the excretion of non-maillard nitrogen in the urine. However, in our study the addition of the water-soluble fraction of the crust to the crumb only slightly affected the digestion of the protein and did not significantly reduce the BV of the crumb. The water-soluble fraction constituted 16.3% of the dry weight of the crust but only 13% of the nitrogen. If all non-amino acid, low-molecular N-compounds from muscle tissues (essentially creatine, inosine and nucleotids according to Lawrie (11) were extracted they should contribute with about one third of the nitrogen of the extract. In addition, low-molecular-weight water-soluble reaction compounds from the bread crust powder, which were part of the formula, may also be present in this fraction. These compounds would be absorbed and excreted in the urine, giving an apparently reduced BV. When the extract was given together with the crumb, a slightly lowered BV was thus to be expected. Such a tendency could be observed (although not statistically significant, see table 3). It seems thus not likely that the presence of water-soluble Maillard reaction compounds in this fraction caused the decrease of the BV observed in the crust by interfering with the utilization of absorbed protein. However, the standard deviation of the BV measurements were high for all diets with the exception

of casein and it is of little point to further evolve the speculations about mechanisms behind the change of these values.

In conclusion, it appears that the low-molecular-weight compounds present in a glucose-lysine reaction mixture may affect the course of protein digestion in the rat. The effect on the digestion caused by severe heating of a minced meat formula can, on the other hand, not be explained by the potential presence of water-soluble compounds. The low-molecular-weight compounds present in the glucose-lysine reaction mixture and affecting the protein digestion may thus not be present in sufficient amounts in the crust to bring about an effect. They may also be essentially lipophilic and thus escape a water-extraction.

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TABLE 1

Composition of diets for the nitrogen balance studies

Component	Dry weight g/kg	Source
Protein (eqv. to 15 g N)	787	Casein ANRC 30 M from Humko Sheffield Chemicals, New York
Maize starch		AB Risenta, Sweden
Sucrose	100	Swedish Sugar Company AB
Maize oil	50	
Mineral mixture*	48	
Vitamin mixture†	8	Purchased through
Choline chloride	2	hospital pharmacy
Cellulose	5	

* Contained (g): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 8.6, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 32.0, KH_2PO_4 7780, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 4024, CaCO_3 7600, KI 1.6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2000, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 180, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 80, CoCl_2 0.46, NaCl 2382.

† Contained (g): menadione 2.5, thiamin hydrochloride 10.0, riboflavin 10.0, pyridoxin hydrochloride 5.0, calcium pantothenate 25.0, nicotinic acid 25.0, folic acid 1.0, inositol 50.0, p-aminobenzoic acid 5.0, biotin 0.2, vitamin B_{12} 0.015, Cyanocobalamin 0.015, vitamin A0.86, vitamin D 0.025, vitamin E 100, wheat starch 3765.

TABLE 2

Radioactivity in the plasma of rats two hours after ingestion of radiolabelled free lysine, proteinbound lysine and proteinbound cysteine. Values are dpm per ml plasma.

Radioisotop diet	^{14}C		^3H		^{35}S		$^{14}\text{C}/^3\text{H}$		$^{35}\text{S}/^3\text{H}$	
	MW	CV ⁺	MW	CV	MW	CV	MW	CV	MW	CV
^{14}C -lysine and ^{14}C -lysine-egg white										
1.5% LMW-fraction in the diet (7)	7823	28.7	77312	25.7			0.102*	9.8		
Control (6)	9010	35.1	78097	38.0			0.117	12.0		
1.7% HMW-fraction in the diet (10)	4923	9.0	46420	9.9			0.106*	3.3		
Control (10)	4543	11.9	45628	7.6			0.100	2.7		
^3H -lysine-egg white and ^{35}S -cystein-egg white										
1.5% LMW-fraction in the diet (8)			21575	9.0	5010	7.7			0.233	4.7
Control (7)			23252	15.0	5448	14.3			0.235	6.4

* Value significantly different from control ($p < 0.05$, Student's t-test).

⁺ CV = coefficient of variation ($100 \times \text{SD}/\text{mean}$)

Within parenthesis: number of rats

TABLE 3

Nitrogen balance studies with components from fried minced meat loaf.

	TD	BV
Casein (reference)	100.1 (0.82)	89.4 (2.99)
Crust	77.4 (2.44,a)	59.9 (5.49,a)
Crumb	98.0 (0.53,b)	71.4 (6.18,b)
Insoluble residue of the crust	75.5 (2.75,a)	71.2 (6.99,b)
Water-soluble fraction of the crust + crumb	95.6 (1.40,c)	67.2 (4.67,b)

Mean values from five rats in each group. Within parenthesis: S.D.; Means not followed by the same letters are significantly different. ($P < 0.05$, Student's t-test).

TABLE 4

Essential amino acids in the crust and crumb of fried meat loaf, % of protein (Nx6.25).

	Crumb	Crust	Rat requirement [*]
Histidine	3.8	3.6	2.5
Isoleucine	4.7	4.8	4.6
Leucine	8.2	8.3	6.2
Lysine	8.6	7.3	7.5
Total sulfur a.a.	3.8	3.4	5.0
Phenylalanine + tyrosine	8.4	8.4	6.7
Threonine	4.8	4.9	4.2
Tryptophan	-	-	1.25
Valine	5.1	5.1	5.0
Arginine	6.0	5.9	5.0

* From Steinke (8)

Figure caption

Figure 1. Level of activity in the plasma of rats after feeding a diet containing free (^3H) and protein-bound (^{14}C) radiolabelled lysine. Filled symbols: 1.5% of the low molecular-weight (LMW) fraction of the glucose-lysine reaction mixture added to the diet. Open symbols: No addition. Each value is the average from 1-4 rats. .

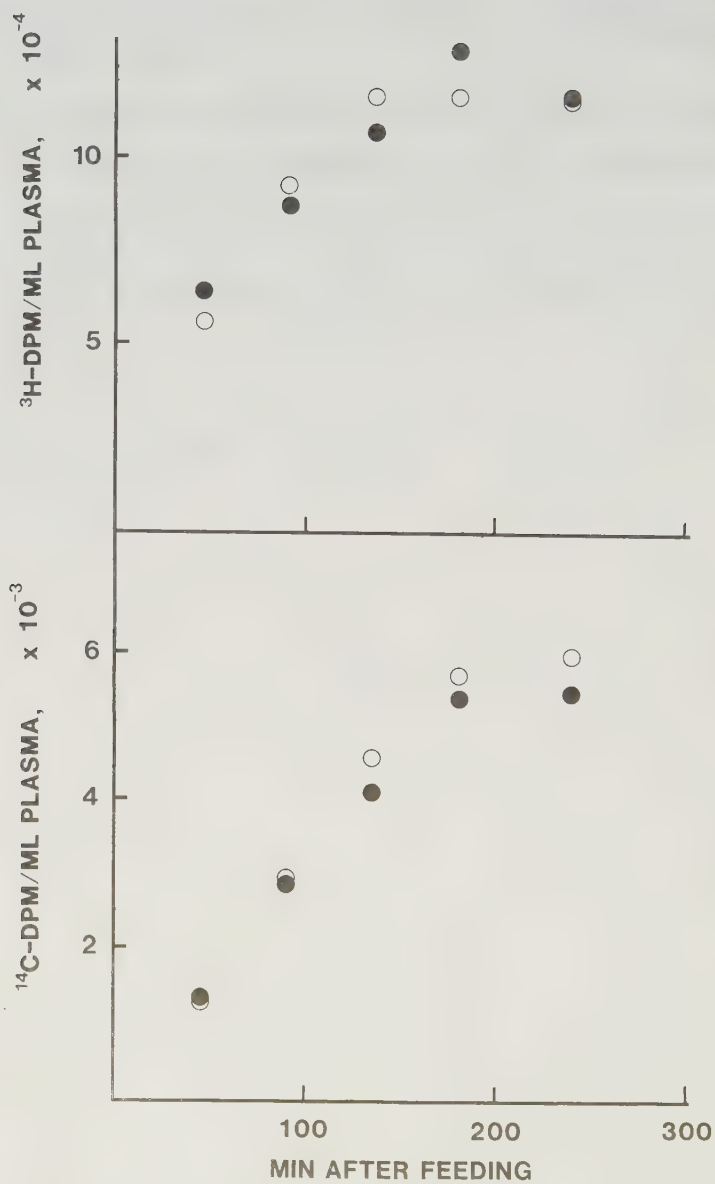


fig. 1

EFFECT OF MAILLARD REACTION PRODUCTS ON PROTEIN DIGESTION.
IN VITRO STUDIES.

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Short title: Maillard reaction and enzyme activities.

Send proof to Rickard Öste

ABSTRACT

The low-molecular fraction of a glucose-lysine reaction mixture, previously shown to affect the *in vivo* uptake of proteins in the rat, was tested for its effect on a number of gastro-intestinal proteases and peptidases *in vitro*.

Of the enzymes tested, carboxypeptidase A was significantly inhibited by 0.5 mg/ml of the Maillard products, and aminopeptidase N (a key enzyme in the brush border hydrolysis of peptides) was strongly inhibited by 0.25 mg/ml.

The inhibition of aminopeptidase N was of the mixed type while the inhibition of carboxypeptidase could not be described by means of classical enzyme kinetics. Gel filtration chromatography indicated that a number of different compounds in the Maillard reaction mixture may inhibit the two enzymes.

The inhibitory effect on these two enzymes may be of importance for the protein uptake *in vivo*.

Key words: Maillard reaction - proteolytic enzymes.

A previous study on the effects of Maillard reaction compounds on the digestion of dietary protein, has indicated (1), that a low molecular-weight (LMW) fraction of a glucose-lysine reaction mixture affects the utilization of dietary protein in the rat. The present paper reports effects of this fraction on the in vitro activities of gastro-intestinal proteolytic enzymes.

It was observed that some of the tested enzymes, notably carboxypeptidase A and the brush border enzyme aminopeptidase N, were inhibited by low concentrations of the LMW fraction.

EXPERIMENTAL

Glucose-lysine reaction mixture

A low molecular-weight (LMW) fraction from a reflux-boiled mixture of equimolar amounts of D-glucose and L-lysine was prepared as described by Øste & Sjödin (1).

Gastric and pancreatic enzymes

Pepsin (EC 3.4.23.2; 2x crystallized, from porcine stomach mucosa), trypsin (EC 3.4.21.4; 2x crystallized, from bovine pancreas), chymotrypsin (EC 3.4.21.1; 3x crystallized, from bovine pancreas), carboxypeptidase A (EC 3.4.17.1; 2x crystallized, from bovine pancreas) and carboxypeptidase B (EC 3.4.17.2; chromatographically pure, from porcine pancreas) were purchased from Sigma Chemical Company, St Louis, Mo., USA. The activities of pepsin with hemoglobin

and trypsin with N-benzoylarginine ethyl ester (BAEE) as substrates were measured as described by Bergmeyer (2). The activities of trypsin and of chymotrypsin with casein as substrates were also measured as described by Bergmeyer (2), but with an incubation temperature of 37°C. The activity of carboxypeptidase A was measured with N-hippuryl-L-phenylalanine as substrate in 0,1 M sodium borate buffer, pH 7,6, 0.3 M in NaCl at a temperature of 30°C. The hydrolysis was monitored by recording the increase in absorbance at 254 nm. The activity of carboxypeptidase B was measured with N-hippuryl-L-arginine as substrate in 0.1 M sodium borate buffer, pH 7,6 and at a temperature of 30°C. Also in this case the hydrolysis was monitored by recording the increase in absorbance at 254 nm.

Enzymes of the intestinal mucosa

Preliminary experiments with brush border peptidases were performed with enzymes solubilized from the microvillus membrane vesicle fraction. Microvillus membranes were prepared from pig mucosa as described by Sjöström et al (3). The enzymes were solubilized by treatment with 1% Triton X-100 for 1 h and the solution centrifuged at 15,000 rpm in a Sorvall SS 34 rotor for 1 h. The resulting supernatant was used as the enzyme solution.

Preliminary experiments with cytosolic peptidases were performed with a preparation obtained as follows: A piece of pig intestine (about 5 cm) was gently stirred at 4°C in 20 ml of a 50 mM Tris-HCl buffer, pH 8.0, for 1 h, after which the solution was centrifuged at 15,000 rpm in a Sorvall SS 34 rotor for 30 min. The supernatant was used

as an enzyme solution.

Kinetic experiments with aminopeptidase N (EC 3.4.11.2) were performed using the enzyme prepared as described by Sjöström et al (3).

The activities of aminopeptidase N, aminopeptidase A (EC 3.4.11.7) and dipeptidylpeptidase IV were analysed as described by Sjöström et al (3), using L-alanyl-p-nitroanilide, L-glutamyl-p-nitroanilide, and glycyl-L-prolyl-p-nitroanilide as substrates, respectively.

Activity of glycyl-leucine dipeptidase (EC 3.4.13.11) and alanyl-proline dipeptidase (EC 3.4.13.9) were assayed by measuring the decrease in absorbtion in the low-ultraviolet range, using glycyl-L-leucine and L-alanyl-L-proline as substrates, respectively, with details as described (4).

Column chromatography

A 2,6 x 90 cm column was packed with Sephadex G-50 (superfine grade, swelled in water). 200 μ l (38 mg dry substance) of a LMW fraction of the glucose-lysine reaction mixture, radiolabelled through addition of 5 mCi (14 C-U)-glucose to the reactants, was placed on the column and eluted with water (eqvilibrated to 4⁰C; flow rate 16.6 ml/h). The UV-absorbance of the eluate was recorded. Fractions of 8.3 ml were collected, lyophilized and redissolved in 1.5 ml of water. The radioactivity of each fraction was assessed by liquid scintillation counting (instrument Mark III, Searle Analytic Inc., Ill). The effects of aliquots of fractions on the activities of carboxypeptidase A and aminopeptidase N were determined.

RESULTS

Effect of the LMW fraction on enzyme activities

The LMW fraction was tested at concentrations between 0.25 - 1 mg/ml in the assay mixtures. Of the gastric and pancreatic enzymes studied, trypsin was slightly inhibited by 0.5 mg/ml, when BAEE was the substrate. The inhibition was non-competitive. The activity of trypsin against a protein substrate, casein, was somewhat reduced by 1,0 mg/ml of the LMW fraction but only at low concentrations of the substrate. Carboxypeptidase A and, to a smaller extent, carboxypeptidase B were inhibited by 0,5 mg/ml of the LMW fraction. The inhibition of carboxypeptidase A did not obey classical enzyme kinetics, as indicated by the Lineweaver Burk plot shown in figure 1. The enzymatic activities were completely restored after dialysis of the reaction mixture. No effect on pepsin or chymotrypsin was observed.

Aminopeptidase N of the brush border membrane was strongly inhibited by 0,25 mg/ml of the LMW fraction. The inhibition was apparently of the mixed type with a strong non-competitive element (see fig 1). The cytosol enzyme glycyl-leucine dipeptidase was inhibited by 0,7 mg/ml. The other enzymes of the mucosa were not affected.

Studies on fractions from column chromatography

The elution profile of the LMW fraction from the glucose-lysine reaction mixture, as monitored by UV-absorbance, is shown in figure 2. The distribution of the label from reactant-glucose is also shown. The chromatography was performed on Sephadex G-50, which has a

separation range of 1,500-30,000 daltons for proteins and 500-10,000 daltons for dextrans. The shape of the profiles of both absorbance and radioactivity indicates that the majority of compounds elute late and have molecular weights in the lower range. The maximum of UV absorbance did not coincide with the maximum of radioactivity.

In figure 2 is also shown the degree of inhibition of carboxypeptidase A and of aminopeptidase N by aliquots of fractions of the eluate. The inhibition of carboxypeptidase A seemed roughly to coincide with the UV absorbance. The inhibition of aminopeptidase N varied considerably from fraction to fraction.

Both carboxypeptidase A and aminopeptidase N were strongly inhibited by a common fraction. This fraction corresponded to a single peak in the UV absorbance curve (indicated in the figure). ^1H -NMR analysis showed that it mainly contained 2-formyl-5-hydroxymethylpyrrole-1-norleucine, a compound isolated from a glucose-lysine reaction mixture by Miller *et al* (5).

DISCUSSION

The LMW fraction from the glucose-lysine reaction mixture affected the activity of some, but not all, of the enzymes involved in protein digestion *in vivo*, at least down to a concentration of 0,5 mg/ml. The question is, whether this partial inhibition might explain the earlier observed (1) reduced plasma level of amino acids derived from dietary egg-white protein in rats which were orally fed a diet containing 1,5% (w/w) of the LMW fraction. In that study, each rat consumed 22,5 mg of the LMW fraction. Assuming a

total volume of digestive juices of 5-10 ml, the concentration in the rat intestine will most likely be high enough to bring about an enzyme inhibition, even with a high degree of absorption of the compounds to non-specific proteins. The inhibitory effects observed in vitro were of the non-competitive or the mixed type. This means that even under conditions of excess substrate concentration, likely to exist at least initially in the lumen of the intestine, an effect on the substrate turnover is to be expected. If the enzyme activities thereby becomes rate-limiting for the overall digestion and absorption, a reduced blood uptake of initially protein-bound amino acids might be the result. However, it is unclear to what extent a partially reduced enzyme activity in vivo will actually lead to an impaired amino acid uptake. The reduced (50-70%) level of pancreatic enzymes in aged rats does not reduce protein digestibility (6). Total exclusion of pancreatic secretion, however, leads to a markedly reduced protein digestibility (6). As for the inhibition of specific enzymes, it has been reported that during in vitro conditions, the single inhibition of one of the enzymes leads to a notable reduction of proteolytic capacity of the mixture of pancreatic enzymes (7). This was observed also when the exopeptidases were inhibited.

Of the pancreatic enzymes investigated in the present study, carboxypeptidase A, carboxypeptidase B and trypsin were affected by the Maillard reaction mixture. However, the degree of inhibition was in no case substantial at 0,5 mg/ml and the slight effect on trypsin digestion of casein at 1,0 mg/ml of the reaction mixture could only be observed at low

substrate concentrations. It seemed thus not probable that these effects were sufficient to bring about a notable effect *in vivo*.

The residue from pancreatic digestion consists of, in addition to free amino acids and dipeptidases, a considerable amount of small oligopeptides (8). The epithelial cells of the intestine have been shown to actively absorb amino acids, dipeptides and possibly tripeptides. The extent to which higher oligopeptides may be absorbed has not been clarified (9, 10). Studies by Adibi (11) shows that the brush border hydrolysis is the rate-limiting step in the absorption of a tetrapeptide, tetraglycine, indicating that the higher oligopeptides are not absorbed but have to be further hydrolyzed prior to uptake. The membrane peptidases aminopeptidase N, aminopeptidase A and dipeptidyl peptidase IV are probably those responsible for this final digestion of luminal oligopeptides and thus the complete uptake of a dietary protein. Aminopeptidase N constitute 5-15% of the total membrane protein and should thus be the dominant one of these (3). Studies by Friedrich et al (12-13) indicate that this enzyme in fact plays a central role in the brush border digestion of peptides *in vitro*. This enzyme was strongly inhibited by the LMW fraction.

About 40% of the LMW fraction is absorbed from the small intestine in the rat (unpublished results). A part of the fraction may thus be present in the epithelial cells at the time of intracellular peptide hydrolysis and may affect the final digestion of (di-)peptides, since an effect on an

intracellular dipeptidase (gly-leu dipeptidase) was observed as well.

The results from the studies on chromatographically separated fractions of the Maillard reaction mixture revealed that no single compound could account for the inhibition of aminopeptidase N or carboxypeptidase A. In addition, the profiles of inhibition of the enzymes by the various fractions were different (see figure 2). This indicates that the compounds may affect the two enzymes to a different extent. The radioactivity in each fraction may roughly be considered proportional to the total amount of Maillard reaction compounds. The profiles of inhibition did not coincide with radioactivity, hence the inhibitory effect was not a feature common to the majority of compounds in the mixture. The nature of the specific compounds that in fact have inhibitory effects will be reported.

The present study supports the assumption that low molecular-weight glucose-lysine reaction compounds influence the uptake of protein in rats (1) due to an inhibition of intestinal proteolytic enzymes. It seems most likely that this effect is mainly the result of specific inhibitions of enzymes of the small-intestinal mucosa, rather than an interference with the pancreatic enzymes. In particular, the inhibition of aminopeptidase N might be crucial.

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Figure captions.

Figure 1. Effect of the low molecular-weight (LMW) fraction of the glucose-lysine reaction mixture on enzyme kinetics. Reciprocal plot of velocity (v) versus substrate concentration (2). Upper figure: Hydrolysis of N-hippuryl-L-phenylalanine by carboxypeptidase A (in 0.1 M Na-borate, 0.3 M NaCl, pH 7.6 at 30°C). Filled symbols: Addition of 0.5 mg/ml of the LMW fraction. Open symbols: No addition. Lower figure: Hydrolysis of L-alanyl-p-nitroanilide by aminopeptidase N (in 0.05 M Tris-HCl, pH 7.3 at 37°C). Filled symbols: Addition of 0.25 mg/ml of the LMW fraction (K_m 0.7 mM, V_{max} 40 mM $\cdot$ min⁻¹). Open symbols: No addition (K_m 0.5 mM, V_{max} 80 mM $\cdot$ min⁻¹).

Figure 2. Elution profile of a Sephadex ^RG-50 ('superfine' grade) gel permeation column of low molecular-weight glucose-lysine reaction products (LMW fraction). UV absorbance was recorded. Shaded area: relative distribution of a ¹⁴C-label on the reactant glucose. Inhibition of aminopeptidase N (●) and carboxypeptidase A (▲) by aliquots of eluent fractions was measured as the reduction of reaction velocity at a 1.0 mM concentration of substrate for both enzymes.

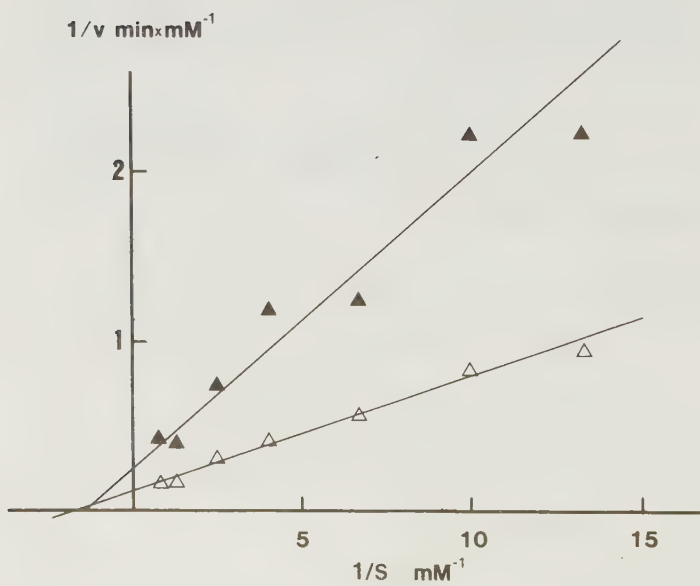
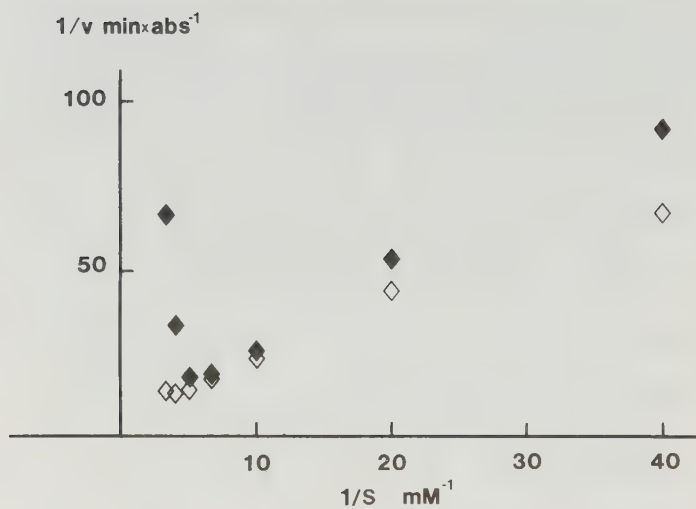


fig. 1

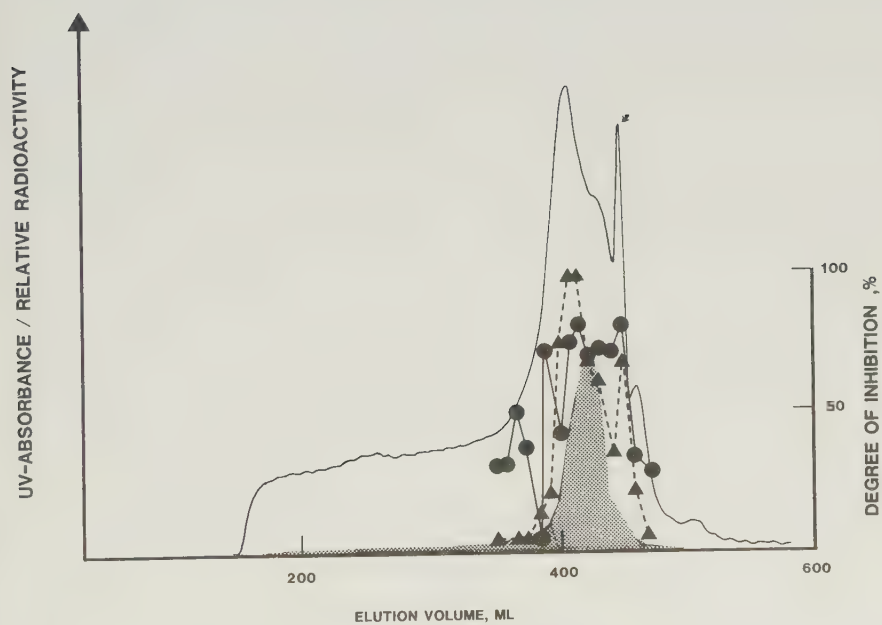


fig. 2

EFFECT OF MAILLARD REACTION PRODUCTS ON PROTEIN
DIGESTION. IDENTIFICATION OF ACTIVE COMPOUNDS.

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ABSTRACT

The effects of pure compounds, similar or identical to those formed in the Maillard reaction, on the activity of carboxypeptidase A and the brush border enzyme aminopeptidase N were studied. A number of furans and pyrroles were found to inhibit carboxypeptidase A. The strongest inhibitors had a carboxylic substituent on the furan ring and the effect of these ones could be characterized as an induced substrate inhibition. A few competitive inhibitors of aminopeptidase N were found, the most effective being DL-2-Formyl-5-(hydroxymethyl)pyrrole-1-norleucine. This compounds also inhibited carboxypeptidase A. When fed to rats (3 mg in a 1 gram portion), it reduced the plasma level of lysine from the diet.

INTRODUCTION

In two preceeding publications (1,2) it was reported that a low-molecular weight fraction from a reaction mixture of glucose and lysine affect the digestion of dietary protein in the rat. This reaction mixture also inhibited some of the enzymes involved in protein digestion in vitro, notable carboxypeptidase A (E.C. 3.4.12.2) and aminopeptidase N (E.C.3.4.11.2). The aim of the present study was to identify compounds formed by the Maillard reaction that inhibits these enzymes (2) and to study whether such compounds could affect the in vivo uptake of dietary proteins.

2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine (compound 1 in figure 1) was identified as the main compound in a fraction of the reaction mixture that strongly inhibited both of the above mentioned enzymes (2). In the present investigation the effects of the synthetically prepared compound 1 on these enzymes were studied. Since both enzymes were inhibited, a systematic study was performed with structurally related compounds. Two of the compounds that were found to be inhibitors were then administered in a low amount to rats together with an experimental double-isotope diet, designed as described earlier (1), to detect effects on the digestion of exogenous protein in vivo.

EXPERIMENTAL

Materials

Compounds (structures and names given in figure 1) 1 (3), 2 (4), 4 (5), 5 (6), 8 (7), 13 (8), 14 (9) and 20 (10) were prepared according to the literature. Compound 7 was obtained by saponification of the ester ethyl 2,5-Dimethylpyrrole-1-acetate prepared as described (11). Compound 12 was prepared from commercial 5-methyl furfural by the Canizzaro reaction. The other compounds shown in figure 1 as well as all other chemicals used were commercial samples of analytical grade.

Each compound was dissolved in a small quantity of methanol prior to assaying its enzyme inhibitory effects. This addition of methanol did not affect the enzymes at the conditions of the assays.

Enzyme assay

Sources of the enzymes and the assay procedures for carboxypeptidase A and aminopeptidase N were as described earlier (2). The effects of the compounds on enzyme activities were tested with 0,1-0,8 mM solutions of each compound in the assay mixtures.

Animal experiments

A balanced, radioactive diet containing L-(4,5-³H)-lysine (80 Ci/mmol) and L-(U-¹⁴C) lysine-egg white (10,500 dpm/mg) was prepared as described (1), with the exception that all protein was supplied as egg-white protein. The radioactivity in the diet was 6950 dpm of ³H/mg and 1090 dpm of ¹⁴C/mg. It was used as control

diet and for the preparation of the experimental diets. These were obtained by the addition of compound 1 or compound 11 to a concentration of 3 mg/g.

Individually caged, overnight fasted male Sprague-Dawley rats, 100-110 g, were in the morning fed one of the experimental diets or the control diet. After three hours, the rats were anaesthetised and blood was obtained by heart puncture and sampled into heparinized test-tubes. The radioactivity in plasma was determined by liquid scintillation counting after mixing with Instagel (Packard Instr. Co., Ill).

RESULTS

A number of the tested heterocyclic compounds inhibited the activity of carboxypeptidase A in a way that could not be explained by conventional kinetic theory of enzyme - inhibitor interactions (12). The inhibition by a certain concentration of the inhibitor increased strongly when the substrate concentration was raised, i.e. a substrate inhibition was induced by the inhibitor (figure 2). Attempts to fit the measurements to a straight line in the Lineweaver-Burk plot were unsuccessful. However, as judged by comparison of the shape of the graphs of velocity versus substrate concentration, the strength of inhibition of the different compounds could be roughly graded as shown in figure 1. The gradation could be related to the structure with notable features as follows:

- Furans that have a carboxylic group as substituent were strongly inhibitory.

- The two-fold symmetric compounds 4, 7, and 8 had low or no inhibitory effect regardless of the substituent polarity.
- Compound 1, having a norleucine residue bound to the pyrrole nitrogen, inhibited somewhat stronger than the N-unsubstituted compound 2.

Of the tested compounds, 1, 7, and 8 inhibited aminopeptidase N significantly. The inhibitions were competitive and the calculated inhibition constants are given in figure 1. In addition, a few weaker inhibitors were found (cpd's 11, 12, and 19). We also observed that compound 1, and 7 were the only inhibitors stronger than lysine, which was a competitive inhibitor with a K_i of approximately 2 mM.

The results from the in vivo experiments on rats with compounds 1 and 11 are shown in table 1. A small but statistically significant reduction of the plasma level of both ^3H and ^{14}C was observed with 1 in the diet, when compared to the control. No significant effect was observed with compound 11. The $^{14}\text{C}/^3\text{H}$ ratio was about the same in all three groups. The results suggest a reduced utilization of dietary protein-bound and free lysine induced by the presence of 1 in the diet.

DISCUSSION

The Zn-containing exopeptidase carboxypeptidase A catalyses the hydrolysis of peptide bonds next to the carboxyl-terminal end of peptides and proteins, preferentially when the side-chain of the carboxyl-terminal amino acid is hydrophobic as in phenylalanine, tryptophan or leucine (13). This specificity may be attributed to the presence of a hydrophobic pocket in the active site of the enzyme (14). A number of inhibitors have been reported. These may be aromatic or aliphatic, hydrophobic or partly hydrophilic compounds with or without an adjacent carboxyl group (15, 16, 17). The type of inhibition differs with the type (ester or peptide linkage, 18) and the size (19) of the substrate molecule. Some compounds possess both inhibitory and activating properties (16).

The previously reported effects of a glucose-lysine reaction mixture on protein utilization in vivo and in vitro suggests the presence of low-molecular weight enzyme inhibitors in this mixture (1,2). During the Maillard reaction numerous hydrophobic, aromatic and aliphatic, compounds are formed from dehydration products of the initially formed, highly hydrophilic reaction products of reducing carbohydrates and amines (20). It seemed thus not unreasonable to expect that some of these compounds are inhibitors of carboxypeptidase A. For example, the formation of keto acids is consistent with the established lines of reactions and levulinic acid have been isolated in Maillard reaction mixtures (21, 22). A number of keto acids, including levulinic acid, have been shown to inhibit carboxypeptidase A (17). However, as was reported (2), carboxypeptidase A was inhibited by a number of fractions of a

gel-chromatographed glucose-lysine reaction mixture, the degree of inhibition being roughly proportional to UV-absorbance. Thus the presence of aromatic inhibitors was indicated and one compound, DL-2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine (1), was established as the major one in a strongly inhibiting fraction (2). In the present study we have shown that this compound inhibited carboxypeptidase A, as did a number of other heterocycles. Among these, compounds 1, 2, 5, 6, 9, 11, 13, 15, and 17 are formed in the glucose-lysine reaction (3). Compounds 3 and 10 are formed in the Maillard reaction if a pentose is present. Compound 16 has been found in a lactose-casein browning system (23). The evidence thus show that aromatic Maillard reaction compounds may be inhibitory to carboxypeptidase A.

The kinetic parameters of the inhibition could not be calculated. In view of the facts that the hydrolysis by carboxypeptidase A of N-substituted dipeptides does not follow the classical Michael-Menten kinetics (24), and that the course of events during catalysis is assumed to be of a multi-step nature (25), this may not be too surprising. The substrate inhibition is in fact a feature observed with at least some of the often employed substrates of carboxypeptidase A (24). However, it was outside the scope of the present investigation to precisely describe the nature of the inhibitor-enzyme interactions and the results will only be considered in relation to the nature of the Maillard reaction.

Furans and pyrroles of the principle structure shown in figure 1 (structure 'a'), are commonly found products of the Maillard reaction. The furans are generated also at the caramellization reaction of pentoses and hexoses in the absence of

amines, although the presence of amines catalyses their formation (26). The most notable inhibition was observed with 5-(hydroxymethyl)-2-furanoic acid (cpd 13) which at 0.1 mM concentration abolished the activity of carboxypeptidase A at high substrate concentration (figure 2). It may be formed from 5-(hydroxymethyl)-2-furaldehyd (cpd 9) during conditions favouring oxidation (27). The latter compound, found to be a weak inhibitor, is one of the major components formed during non-enzymatic browning in food.

The simple pyrroles with unsubstituted nitrogen and the pyridinols (cpd's 2, 3, 5, 6, 14 and 15) are generally assumed to be formed via a reaction pathway that includes Strecker degradation of free amino acids (28). Since the concentration of free amino acids are low in most foodstuffs, the majority of amino groups that may participate in the Maillard reaction probably are those of the protein. Thus the formation of N-unsubstituted pyrroles and pyridinols may be less frequent in real food system. They cannot be completely excluded, however, since during the heating of proteins ammonia is liberated (29) and may form N-unsubstituted heterocycles via direct condensation with carbonyl compounds.

The results showed that the N-substituted pyrrole 1 was a somewhat more effective inhibitor than the unsubstituted analogue 2. This is of particular interest since this type of pyrroles may be readily formed in comparatively high amounts when the reacting amino compound cannot participate in the Strecker degradation, as has been shown by model studies with glucose and methylamine or glycine (8,22). Thus such pyrroles may be formed and may actually be linked to protein-N in foods and eventually be liberated during digestion.

The formation of pyrroles and pyridinols during the Maillard reaction are not only dependent on the reactants but also on the reaction conditions. As shown in model studies (28), the concentration of pyrroles pass through a maximum during the course of the reaction and are consumed during later stages, while the pyridinols are accumulated. The effect of pH on the yield differs with the type of pyrrole or pyridinol being formed and some types are evidently favoured by a pH close to that of foodstuffs (28). However, if not direct analysed, it is impossible, at the present state of knowledge, to tell the amount of a pyrrole or furan in a specific heated foodstuff. From studies of model system (3, 8, 22) it seems that the amount of any specific pyrrole or furan is very low compared to the amount of reacting carbohydrate, but these model system badly correspond to heat-treated foods, when reactant composition and degree of heat treatment are considered.

A few of the compounds were inhibitors of aminopeptidase N. The inhibitions were of competitive nature. Of the effective compounds, only 1 and 11 have been found in the Maillard reaction. These as well as the weak inhibitors 11, 12 and 19 have all a free carboxylic acid group. Carboxylic groups found in Maillard reaction products may originate from a reacting free amino acid, though its carboxylic group should preferably be lost as carbon dioxide in the Strecker degradation. However, in the protein the carboxylic acid forms part of the peptide linkage and is exposed only after digestion. It is thus possible that compound's such as 1 may be formed from protein-bound lysine and may subsequently be liberated in the intestine. Carboxylic groups may otherwise be formed in the Maillard reaction by direct oxidation of aldehydes or possibly through hydrolytic or oxidative fission of dicarbonyls (22, 30).

To what degree an inhibitor of aminopeptidase N may lead to a reduced protein digestion was tested in the in vivo study with radiolabelled free (^3H) and proteinbound (^{14}C) lysine in the diet. The addition of compound 1 to the diet lead to a reduced level of both labels in the 3 hours' plasma. This compound inhibited both aminopeptidase N and carboxypeptidase A in vitro. With the administered amount of this compound (3 mg/rat) we assumed that these enzymes should be affected during the in vivo conditions as well. The reason for the observed effect cannot, however, be completely explained by an effect on these enzymes during the digestion, since the ratio of ^{14}C to ^3H did not change significantly, i.e. free lysine in the diet was affected to about the same extent as protein-bound lysine. The result is, however, reasonable if there also was, in addition to an inhibition of the protein digestion, an effect on the intestinal uptake of lysine. Structurally, compound 1 is a neutral amino acid. Nevertheless, it may interfere with the active transport of lysine into the enterocytes, as such an effect has been observed with the common neutral amino acids (31).

Further we want to point out that an effect on the metabolism of lysine after absorption could affect both labels in plasma to the same degree and give the results shown.

We also fed rats the radioactive diet together with compound 11, that was a stronger inhibitor of carboxypeptidase A, and a weaker one of aminopeptidase n, compared to compound 1. The mean values of both levels of labels were in this case also lower than the control values but the differences were not statistically significant. The results indicates that, if there is an effect of compound 11, it should be a small one.

An earlier study by us (1), performed with the double isotope technique used here, indicates that a low-molecular weight glucose-lysine reaction mixture affects the digestion of protein in vivo. We also showed that this mixture inhibits aminopeptidase N and carboxypeptidase A (2). The inhibition of aminopeptidase N observed in that study was of the mixed type and that of carboxypeptidase A was similar to the type of inhibition we observed with pure compounds in this study. The amount of added inhibitor in the present in vivo study was 0.3% (dry weight) of the diet to be compared with 1.5% of the low-molecular weight glucose-lysine reaction mixture in the earlier study. However, the capacity to competitively inhibit aminopeptidase N of the intestine in these experiments should have been considerably stronger with compound 1 than with the glucose-lysine reaction mixture. Still the effect on the digestion of dietary proteins seems more pronounced with the reaction mixture than with the pure compound 1. However, the inhibition of aminopeptidase N caused by this mixture also had a strong non-competitive element. It may be that this non-competitive inhibition is of importance for the effect in vivo.

In the search for non-competitive or mixed-type inhibitors of aminopeptidase N, we studied a number of fractions from a glucose-lysine reaction mixture that had been examined with the primary purpose to isolate and identify new reaction compounds (3). However, no such inhibitor could be found.

In conclusion, we have found a number of heterocyclic compounds formed in the Maillard reaction that are potent inhibitors of carboxypeptidase A. A few inhibitors of aminopeptidase N were also found, of which at least two are formed in the Maillard

reaction. The in vivo experiments indicate that such compounds may influence the utilization of dietary proteins.

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TABLE 1

The radioactivity in plasma of rats three hours from feeding an experimental diet containing radiolabelled free (^3H) and protein bound (^{14}C) lysine with or without the addition of single Maillard reaction products.

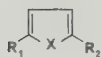
	<u>(^3H)</u>	<u>(^{14}C)</u>	<u>($^{14}\text{C}/^3\text{H}$)</u>
Control diet (7)	29.5 $\pm$ 1365	12.646 $\pm$ 940	0.438 $\pm$ 0.020
Addition of compound <u>1</u> (7)	26.307 $\pm$ 2186*	11.589 $\pm$ 819*	0.427 $\pm$ 0.037
Addition of compound <u>11</u> (7)	27.877 $\pm$ 1871	11.860 $\pm$ 720	0.442 $\pm$ 0.031

Values are dpm/ml. Within parenthesis: number of rats. *Significantly different from control ($p < 0.05$, Student's t-test).

FIGURE CAPTIONS

Figure 1. Structures, names and inhibitory capacity of various compounds formed in, or similar to those formed in the Maillard reaction. The strength of inhibition of carboxypeptidase A is proportional to the numbers of asterisks (see text). A minus indicates no inhibition. NA: not assayed. Conditions of enzyme assays as reported (2).

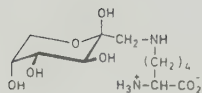
Figure 2. The effect of additions of cpd's 10 (0.4 mM,★), 13 (0.1 mM,✕) or 15 (0.4 mM,○), on the velocity of hydrolysis of N-hippuryl-L-phenylalanine by carboxypeptidase A in 0.1 M Na-borate, pH 7.6, 0.3 M NaCl at 30°C.●: no addition.



(a)



(b)



20

COMPOUND	STRUCTURE		NAME	INHIBITION		
				CARBOXY- PEPTIDASE A	AMINO- PEPTIDASE N	
(a)						
<u>1</u>	$N(CH_2)_4CH(NH_2)CO_2H$	$\begin{matrix} R_1 \\ -CHO \end{matrix}$	$\begin{matrix} H_2 \\ -CH_2OH \end{matrix}$	DL-2-FORMYL-5-(HYDROXYMETHYL) PYRROLE-1-NORLEUCINE	**	COMPETITIVE, $K_I=0.2$ mM
<u>2</u>	NH	-CHO	-CH ₂ OH	5-(HYDROXYMETHYL)PYRROLE-2- CARBOXALDEHYDE	*	-
<u>3</u>	NH	-CHO	-H	PYRROLE-2-CARBOXALDEHYDE	**	-
<u>4</u>	NH	-CHO	-CHO	PYRROLE-2,5-DICARBOXALDEHYDE	-	-
<u>5</u>	NH	-CHO	-CH ₃	5-METHYLPYRROLE-2-CARBOXALDEHYDE	*	-
<u>6</u>	NH	-COCH ₃	-H	2-ACETILPYRROLE	**	-
<u>7</u>	NCH ₂ CO ₂ H	-CH ₃	-CH ₃	2,5-DIMETHYLPYRROLE-1-ACETIC ACID	*	COMPETITIVE, $K_I=0.6$ mM
<u>8</u>	NCH ₂ CO ₂ H	-H	-H	PYRROLE-1-ACETIC ACID	-	COMPETITIVE, $K_I=4$ mM
<u>9</u>	O	-CHO	-CH ₂ OH	5-(HYDROXYMETHYL)-2-FURALDEHYDE	*	-
<u>10</u>	O	-CHO	-H	2-FURALDEHYDE	**	-
<u>11</u>	O	-CO ₂ H	-H	2-FUROIC ACID	***	WEAK
<u>12</u>	O	-CO ₂ H	-CH ₃	5-METHYL-2-FUROIC ACID	***	WEAK
<u>13</u>	O	-CO ₂ H	-CH ₂ OH	5-(HYDROXYMETHYL)-2-FUROIC ACID	***	-
(b)						
	$\begin{matrix} R_1 \\ R_2 \\ R_3 \end{matrix}$					
<u>14</u>	-H	-CH ₃	-OH	2-METHYL-3-PYRIDINOL	-	-
<u>15</u>	-CH ₃	-H	-OH	6-METHYL-3-PYRIDINOL	*	-
<u>16</u>	-H	-CH ₃	-H	2-METHYLPYRIDINE	*	NA
<u>17</u>				LEVULIC ACID	*	-
<u>18</u>				PROPIONIC ACID	-	NA
<u>19</u>				α -HYDROXY PROPIONIC ACID	NA	WEAK
<u>20</u>				1-DEOXY-1-2-N-(L-LYSINO)-D- FRUCTOSE	-	

fig. 1

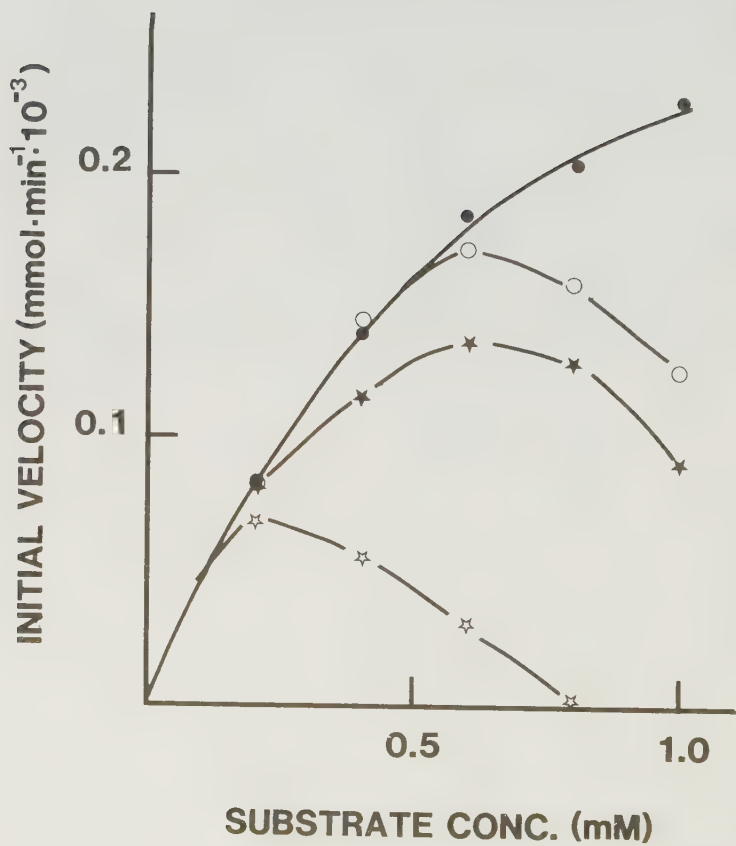


fig. 2

EFFECT OF MAILLARD REACTION PRODUCTS ON CARBOHYDRATE
UTILIZATION. STUDIES IN VITRO AND IN VIVO.

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ABSTRACT

The effect of Maillard reaction products on the digestion of carbohydrates was studied. Low-molecular weight compounds from a glucose-lysine model reaction mixture were weak competitive inhibitors of lactase, invertase, maltase and trehalase in vitro, when tested in concentrations of 5-10 mg/ml. High-molecular weight compounds from the reaction mixture were strong inhibitors of the same disaccharidases at considerably lower concentrations (1.4-2.8 mg/ml). However, when fed to growing rats in amounts up to 40 mg, the high-molecular weight compounds failed to reduce the uptake of intubated lactose or sucrose.

INTRODUCTION

The handling of foodstuffs prior to consumption may include treatments leading to the formation of new molecules, i.e. compounds that are not naturally present in the fresh food. One of the most important mechanisms behind these effects is the Maillard reaction between amino compounds and reducing carbohydrates. This reaction, leading to the formation of strongly coloured products that give the characteristic colour and flavour to fried or baked food, has been the subject of studies from chemical, technological, nutritional and physiological points of view (Eriksson, 1981; Waller & Feather, 1983). However, in spite of its wide occurrence in prepared foods, knowledge of its health implications is still limited.

In a recent study (Öste & Sjödin, 1984, and results to be published) on the effects of Maillard reaction products on the digestion and uptake of dietary proteins, it was observed that some compounds formed in a glucose-lysine model reaction mixture inhibited the action of intestinal peptidases. The present study was performed in order to examine whether Maillard reaction products had any effect on the activity of enzymes involved in the digestion of dietary carbohydrates (for a review of carbohydrate digestion, see Dahlqvist, 1978). Since such an effect was observed *in vitro*, absorption tests of carbohydrates on rats were conducted as well. These tests, however, did not reveal any effect on the uptake of dietary carbohydrates *in vivo*.

EXPERIMENTAL

Model Maillard reaction mixtures

A low-molecular weight (LMW) fraction and a high-molecular weight (HMW) fraction from a glucose-lysine reaction mixture were prepared as described previously (Üste & Sjödin).

Enzyme assays

Alfa-amylase (E.C.3.2.1.1). A stock solution of salivary amylase was prepared by diluting 1 ml of saliva with 40 ml 50% glycerol. Before collecting the saliva the mouth was thoroughly rinsed with water. The amylase activity was measured with soluble starch nach Zulkowsky (from Merck, W.-Germany) as the substrate. A 0.05 M K-Na-phosphate buffer, pH 6.9, with 0.04% NaCl was used to get optimal conditions for the enzyme (Dahlqvist, 1962). The stock solution was adjusted to contain 12 IU/ml (1 IU = 1 μ mol maltose liberated/min). The rate of starch digestion was measured by following liberated reducing groups with 3,5-dinitrosalicylate (DNS) reagent (Hostettler et al, 1951). Maltose was used as the standard. One ml enzyme solution was incubated with 1 ml substrate (1-9 mg starch) at 37°C. The incubation time was kept short to get a low degree of hydrolysis and thus avoid product inhibition. After 6 min 2 ml of DNS reagent were added to the incubation mixture, which immediately inactivated the amylase. A substrate/enzyme blank was obtained by mixing the substrate with the DNS reagent before adding the enzyme.

Disaccharidase. Activities of lactase (E.C.3.2.1.23), maltase (E.C.3.1.1.20), isomaltase (E.C.3.2.1.10), sucrase (E.C.3.2.1.48) and trehalase (E.C.3.2.1.28) were measured on perorally obtained biopsy specimens of human small intestine mucosa as described by Dahlqvist (1968) with the following modification (Dahlqvist, 1984): the TGO reagent

was obtained by dissolving 5,6 g Glox (Kabi Diagnostica AB, Stockholm, Sweden) in 100 ml 0,5 M tris buffer, pH 7.0.

Animal experiments

General performance. Absorption tests were performed on growing male Sprague-Dawley rats weighing 150-180 g. The uptake of sucrose and of lactose was studied by orally intubating the lightly anaesthetized (diethyl ether) fasting (over-night) rats with a 4 ml solution of 760 mg sucrose or 400 mg lactose. After the time allowed for absorption, 0.5, 1, 2, 3, 4 or 6 (for lactose) hours, the rats were narcotized with diethyl ether, the abdomen was opened and the stomach, upper, middle and lower third of the small intestine and the colon and caecum were removed and collected as segments. Each segment was put into a test-tube with a small amount of aq. dest, and immediately placed in a boiling water-bath for two minutes before freezing.

The sum of sucrose, fructose and glucose was determined in the samples from the rats given sucrose by assay of reducing power after enzymatic hydrolysis of the sucrose. Total anthron-reactive carbohydrates were determined in the samples obtained from the rats given lactose.

The rat experiments were performed with or without the addition of the HMW fraction to the carbohydrate solution. In some experiments the HMW fraction (1 ml water-solution) was intubated separately prior to intubation of the lactose solution.

Handling of intestinal segments. Each segment was homogenized (Ultra-Turrax homogenizer) at low temperature (ice-bath) and diluted to 100 ml with aq. dest. Four ml of the homogenate were deproteinized by adding 1 ml 0.3 N Ba(OH)_2 and 1 ml 5% ZnSO_4 (Somogyi, 1945). After centrifugation (1000 g, 10 min) the supernatant was recovered and subjected to carbohydrate analysis as described.

Analysis of total carbohydrates after invertase treatment. Part of the deproteinized supernatant (0.5-1.0 ml) was diluted to 1.0 ml with aq. dest and 1.0 ml of a solution of invertase (2.5 mg/ml B-fructosidase from yeast, purchased as powder from Boehringer Mannheim GmbH, West-Germany, in 0.2 M Na-acetate, pH 4.75) was added. After incubation at 30°C in 60 min, 2.0 ml of the DNS reagent was added and the solution was brought to 100°C for 10 min. 20 ml aq. dest was added and the colour development was measured (room temperature) at 530 nm against a reagent blank. Total reducing carbohydrates in the samples were expressed as glucose equivalents.

Analysis of total carbohydrates with anthron. 0.05-0.5 ml of the deproteinized homogenate was diluted to 2.0 ml and placed in an ice-bath. Four ml anthron reagent (300 mg anthron per 100 ml conc. H_2SO_4 , see Scott & Melvin, 1953), chilled in ice-bath, were slowly added. The mixture was boiled 7.5 min. in a water-bath, and the colour development was measured at 625 nm against a reagent blank. The total amount of anthron-reactive carbohydrates was calculated after analysis of standard solutions of lactose.

RESULTS AND DISCUSSION

Table 1 shows the effect of the glucose-lysine reaction mixtures on the kinetics of the carbohydrate hydrolyzing enzymes. The values of K_m and V_{max} were obtained from Lineweaver-Burk plots (Dixon & Webb, 1964). Amounts of 5-10 mg/ml of the LMW fraction in the assay mixture inhibited the disaccharidases, the inhibition being competitive or of the mixed type. This concentration range was of the same magnitude as that of the substrate and since the inhibition was relatively weak the effect is probably

of little practical significance. It is known that polyols may competitively inhibit disaccharidases, the strength being dependent on the configuration of the polyol (Kelemen & Whelan, 1966; Conclue & Lewy, 1957). During the initial stages of the Maillard reaction, various polyols are formed as the result of condensation of the carbohydrate moiety with the amino compound and a subsequent partial dehydration. During later stages, further dehydration removes more hydroxy groups and gives rise to various aromatic compounds. However, if present, the polyols formed at least initially during the Maillard reaction may very well be responsible for the observed competitive inhibition.

The intense colour of the HMW fraction limited the concentration that could be tested with reasonable accuracy in the assay. In spite of this the presence of 1.4-2.8 mg/ml of this fraction in the assay strongly inhibited all the disaccharidases. The inhibition was of the mixed type with a pronounced competitive element. Invertase and lactase were most affected. Trehalase was inhibited as well but the obtained substrate concentration/velocity values did not fit to a straight line in the Lineweaver-Burk plot.

The complete structure of the polymeric compounds (melanoidines) formed during the Maillard reaction is unknown. However, various functional groups and partial structures have been detected, including hydroxy groups as well as more dehydrated, aromatic parts (Pernemalm, 1978; Kato & Tsuchida, 1981). It is thus not unreasonable to assume that these polymers have both polyolic and more lipophilic regions. Indeed, a repeating unit in the polymer involving polyolic structures have been proposed (Kato & Tsuchida, 1981). In addition, the melanoidines are known to adsorb to proteins (Horikoshi & Gomyo, 1976). With both polyolic structures and adsorptive capacities, the compounds of the HMW

fraction may in fact possess such inhibitory properties as we observed with the disaccharidases.

Salivary α -Amylase was not inhibited by either the LMW or the HMW fraction (the relatively low concentration of them in the assays were due to the interfering colour). Since the properties of salivary amylase are close to those of pancreatic amylase (Meyer et al, 1948, the enzymes are probably identical), it is reasonable to extrapolate the results and assume that the intestinal digestion of starch in general should not be affected by the presence of Maillard reaction compounds.

The intestinal uptake of sucrose and of lactose in the rat with (1%, w/w) and without the HMW fraction added is shown in figure 1. No effect on the time curve of the absorption or the total amount absorbed could be observed. The concentrations of the HMW fractions in the intubated solutions were low compared to the carbohydrates but still high enough (a total amount per rat of 7.6 mg in the sucrose study and 4 mg in the lactose study) to most likely bring about an inhibition *in vivo*, since the observed *in vitro* effect was very strong. However, in rat as well as in man the hydrolytic capacity of sucrose exceeds the transport capacity for the monosaccharides formed (Herman, 1976; Dahlqvist & Thomson, 1963). Thus a partial inhibition might not be detected with the present methodology and may in that case also be of little practical significance. On the other hand, the intestinal lactase activity is rate-limiting for the utilization of dietary lactose, both in most adult humans (Dahlqvist, 1962) and in the rat (Dahlqvist & Thomson, 1964). This means that if the lactase activity was affected, this should be reflected in the amount of remaining lactose in the intestine. No such effect was observed in our study. Possible explanations to this may include too low a concentration of the inhibiting compounds of the HMW fraction at the site of hydrolysis. However, if the volume of the intestinal

content was between 5-10 ml, the concentration of the HMW fraction should have been about 0.4-0.8 mg/ml, or enough to get a notable inhibition. In addition, rats receiving considerably more of the HMW fraction (up to 40 mg) were still capable of absorbing lactose to the same extent as control rats, as judged from the amount of lactose remaining in the gastro-intestinal tract two hours from feeding (table 2).

It thus seems evident that the lack of an *in vivo* effect is not the result of too low concentrations of these compounds in the rat intestine. However, it is still possible that the inhibitor concentration at the digestive site and moment were not sufficient to bring about an effect. Horikoshi et al (Horikoshi et al, 1981) reported that the polymers of a glucose-glycine reaction mixture were strongly adsorbed to the mucus membrane of the small intestine in the rat. If such an adsorption occurred in our experiments, the polymers might have been excluded from an action of competitive inhibition of the enzymes. The same authors also reported that the brown polymers after feeding by intubation formed a hard gel in the stomach. Such an effect is also in concordance with our observation that radiolabelled high-molecular weight compounds from a glucose-lysine reaction mixture to a large extent still remained in the stomach eight hours after intubation (Nair et al, 1981). If so, the polymers and the disaccharide solution might have left the stomach at different occasions and the absorption of lactose would then proceed unaffected. To test if such an effect was present in our experiments, we fed rats the HMW fraction 2, 4, 6 or 16 hours in advance of the lactose solution. However, as seen in table 3, the lactose absorption was still unaffected.

The results suggest that there is no effect *in vivo* on the lactase activity in the rats. The HMW fraction may mediate its effect *in vitro* through properties of the free enzyme that are not present when the

enzyme is membrane-bound in the intestine. Another, possibly more likely explanation could be the presence of a diffusion barrier close to the epithelial cell membrane in the intestine, through which the substrate molecule has to penetrate before reaching the enzyme. A barrier in the form of an "unstirred layer of water" has been observed to greatly influence the rate of diffusion of molecules to the microvillus membrane and into epithelial cells in vitro (Wilson & Dietschy, 1974). The effect was more pronounced with larger molecules than with smaller ones. Molecules of the HMW fraction have a size exceeding 6,000-8,000 dalton (3) and may thus exhibit a considerable restraint to diffusion through this layer, compared to the lactose molecule.

In conclusion, the presented results showed that low-molecular weight compounds from a glucose-lysine reaction mixture were weak inhibitors of the disaccharidases of the gastro-intestinal tract. High-molecular weight compounds, on the other hand, were potent inhibitors in vitro, especially of lactase and invertase. During in vivo conditions, the same compounds failed to affect the uptake of dietary disaccharides.

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CARBOHYDRATE-HYDROLYZING ENZYMES IN VITRO.

Enzyme	No addition	Addition of the LMW fract.		Addition of the HMW fract.	
		5 mg/ml	10 mg/ml	0.8 mg/ml	1.4 mg/ml 2.8 mg/ml
α -Amylase					
K_m^{-1} g/l	9.2×10^{-4}	8.5×10^{-4}		11.5×10^{-4}	
V_{max} app/ V_{max}	-	1.0		1.0	
Lactase					
K_m^{-1} [M]	1.5×10^{-2}	3.6×10^{-2}	6.7×10^{-2}		2.0×10^{-1}
V_{max} app/ V_{max}	-	1.1	0.7		0.3
Invertase					
K_m^{-1} [M]	2.5×10^{-2}		3.1×10^{-2}		2.1×10^{-1}
V_{max} app/ V_{max}	-		1.1		0.2
Maltase					
K_m^{-1} [M]	4.3×10^{-3}	6.3×10^{-3}	11×10^{-3}		14.7×10^{-3}
V_{max} app/ V_{max}	-	1.0	0.9		0.8
Trehalase					
K_m^{-1} [M]	2.7×10^{-3}		3.7×10^{-3}		+
V_{max} app/ V_{max}	-		0.9		

+ Trehalase was inhibited by the HMW fraction but the obtained substrate concentration/velocity values did not fit to a straight line in the Lineweaver - Burk plot and K_m or V_{max} could not be calculated.

TABLE 2 EFFECT OF THE INTUBATED AMOUNT OF HIGH-MOLECULAR-WEIGHT
 MAILLARD REACTION PRODUCTS (HMW FRACTION) ON TWO HOURS LACTOSE
 ABSORPTION IN RATS.

HMW fraction % of administred lactoe (400 mg)	Remaining lactose, % of administred dose	
	Stomach	Intestine
0 (10)	0.78±0.44	9.9±2.9
1 (10)	0.57±0.50	9.6±2.3
3 (10)	0.77±0.60	7.3±3.6
10 (8)	0.97±0.49	7.8±2.0

All values are mean $\pm$ SD

Within parenthesis: number of rats

Mean rat weight 133 g

TABLE 3 EFFECT OF A TIME-LAG BETWEEN INTAKE OF HIGH MOLECULAR-WEIGHT MAILLARD REACTION PRODUCTS (HMW FRACTION) AND INTAKE OF LACTOSE ON TWO HOURS LACTOSE ABSORPTION IN RATS.

Time-lag from intake of HMW fraction (20 mg) to lactose intake (hours)		Remaining lactose, % of administered dose (400 mg)	
		Stomach	Intestine
0	(5)	2.4 $\pm$ 1.9	30.1 $\pm$ 6.4
2	(4)	3.7 $\pm$ 2.8	25.9 $\pm$ 9.0
4	(5)	3.4 $\pm$ 2.6	29.3 $\pm$ 9.9
6	(5)	1.9 $\pm$ 1.2	22.9 $\pm$ 6.8
16	(5)	6.3 $\pm$ 2.5	23.8 $\pm$ 6.6

All values are mean $\pm$ SD

Within parenthesis: number of rats

Mean rat weight 136 g

Figure Caption

Figure 1. Rats (150-180 g) were fed (orally intubated) a sucrose (760 mg) or a lactose (400 mg) solution with (filled symbols) or without (open symbols) the addition of 1% high-molecular weight Maillard reaction compounds (HMW fraction). After the time allowed for absorption, the total carbohydrate remaining in the gastro-intestinal tract was analysed. Each point represents the average of two or three rats.

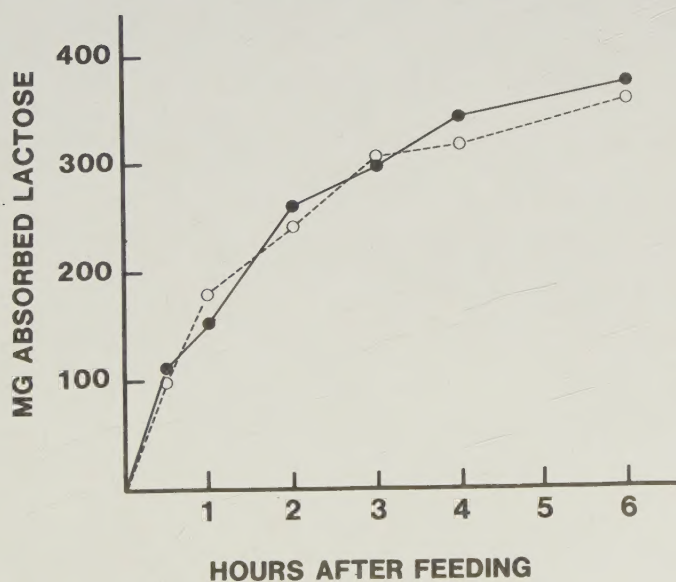
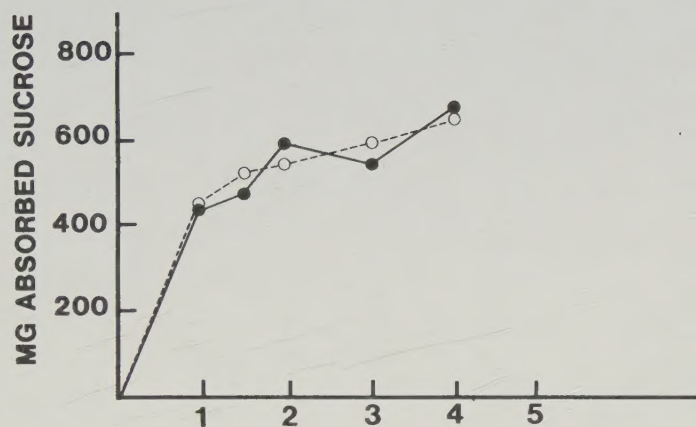


fig. 1

Figure 1 shows the effect of the concentration of the solution on the rate of the reaction. The rate of the reaction increases with the concentration of the solution. The rate of the reaction is highest at a concentration of 0.1 M and decreases as the concentration decreases. The rate of the reaction is lowest at a concentration of 0.01 M.

